ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2004, p. 1681–1687 Vol. 48, No. 5
0066-4804/04/$08.00+0 DOI: 10.1128/AAC.48.5.1681–1687.2004

Pseudomonas aeruginosa LasA Protease in Treatment of Experimental Staphylococcal Keratitis

Irina S. Barequet,[1] Guy J. Ben Simon,[1] Mary Safrin,[1] Dennis E. Ohman,[2] and Efrat Kessler[1]*

Maurice and Gabriela Goldschleger Eye Research Institute, Sheba Medical Center, Tel Aviv University Sackler Faculty of Medicine, Tel-Hashomer 52621, Israel,[1] and Medical College of Virginia Campus of Virginia Commonwealth University and McGuire Veterans Affairs Medical Center, Richmond, Virginia 23298-0678[2]

Received 15 July 2003/Returned for modification 30 November 2003/Accepted 19 January 2004

LasA protease is a staphylolytic endopeptidase secreted by *Pseudomonas aeruginosa*. We have examined the effectiveness of LasA protease in the treatment of staphylococcal keratitis caused by methicillin-sensitive *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) isolates in a rabbit model. Keratitis was induced by intrastromal injection of the bacteria. The eyes were treated topically, and the efficacy of LasA protease was compared to those of lysostaphin (a staphylolytic protease secreted by *Staphylococcus simulans*) and vancomycin. When treatment was initiated early (4 h) after infection, practically all of the MSSA- and MRSA-infected corneas were sterilized by LasA protease, and its efficacy in eradicating the bacteria was comparable to those of lysostaphin and vancomycin. By contrast, most of the control corneas were heavily infected, with median values of 4.5×10^6 (MSSA) and 5×10^5 (MRSA) CFU/cornea ($P < 0.001$). When treatment was initiated late (10 h) after infection, LasA protease reduced the numbers of CFU in both MSSA- and MRSA-infected corneas by 3 to 4 orders of magnitude compared to the numbers of CFU for the controls (median values, 1,380 and 30 CFU/cornea, respectively, for the treated animals compared to 1.2×10^6 and 5×10^5 CFU/cornea for the respective controls [$P = 0.001$]), and it was more effective than vancomycin in eradicating MRSA cells ($P = 0.02$). In both the early- and the late-treatment protocols, the clinical scores for eyes treated with LasA protease were significantly lower than those for the eyes of the corresponding controls and comparable to those for the lysostaphin- and vancomycin-treated eyes. We conclude that LasA protease is effective in the treatment of experimental *S. aureus* keratitis in rabbits and may have potential for the treatment of disease in humans.

Staphylococcus aureus is among the most frequent causes of bacterial keratitis (1, 3). *S. aureus* keratitis is potentially a sight-threatening ocular infection (24, 27); therefore, it requires intensive antibacterial therapy to eliminate rapidly the infecting organism, reduce the inflammatory response, and prevent structural damage to the cornea. Despite the availability of numerous efficient antibiotic agents, management of *S. aureus* infections remains a challenge due to the constantly increasing incidence of clinical isolates resistant to antibiotics (1, 14, 25, 32). Moreover, the recent emergence of *S. aureus* strains resistant to vancomycin (34, 36), the drug of choice for the treatment of infections caused by antibiotic-resistant gram-positive organisms, emphasizes the need for new broad-range and efficient antibacterial agents.

An alternative approach to antibiotic treatment of *S. aureus* infections involves the use of lysostaphin, a 27-kDa staphylolytic endopeptidase that degrades the pentaglycine bridges of the peptidoglycan of *S. aureus* cells (33), as a therapeutic agent. The beneficial effect of lysostaphin has been demonstrated in numerous studies (13, 16, 17, 31, 38). Recently, it was shown to effectively eradicate oxacillin-resistant and vancomycin-intermediate-susceptible *S. aureus* strains in a rabbit model of endocarditis (8, 28), and its ability to kill methicillin-resistant *S. aureus* (MRSA) has been demonstrated in rabbit models of both keratitis (9, 11) and endophthalmitis (10, 11).

LasA protease (also called staphylolysin) is a 20-kDa staphylolytic endopeptidase secreted by *Pseudomonas aeruginosa* (19–21). It cleaves peptide bonds following Gly-Gly pairs in peptides or proteins (22, 35), and as in the case of lysostaphin, its ability to lyse staphylococci results from cleavages within the pentaglycine cross-links in the peptidoglycan of *S. aureus* cells (20, 21). LasA protease can cause lysis of a wide range of *S. aureus* strains (5), and it has also been shown to inhibit the growth of *S. aureus* cells in vitro (26, 29). This favors LasA protease as another useful agent in enzyme-based treatment of *S. aureus* infections. LasA protease is not a major virulence factor in experimental *P. aeruginosa* keratitis (2, 18, 30, 37). Furthermore, topical application of LasA protease on scarified mouse corneas causes little or no damage to ocular tissues (30). Therefore, we have selected an experimental model of *S. aureus* keratitis in rabbits to evaluate the protective effect of LasA protease in vivo. We show that topical treatment of experimental keratitis induced by a methicillin-sensitive *S. aureus* (MSSA) strain, as well as an MRSA strain, reduces the number of *S. aureus* cells in the infected corneas dramatically. Our results support the potential of LasA protease as a therapeutic tool in the management of *S. aureus* corneal infections.

* Corresponding author. Mailing address: Goldschleger Eye Research Institute, Sheba Medical Center, Tel-Hashomer 52621, Israel. Phone: 972-3-535-0392. Fax: 972-3-535-1577. E-mail: ekessler@post.tau.ac.il.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Two clinical isolates of *S. aureus*, an MSSA ocular strain isolated from a human corneal specimen and an MRSA strain isolated from human blood (see the 50% inhibitory concentrations [IC_{50}s] in Table 1), were used to induce experimental keratitis. Both strains were propagated on Mueller-Hinton agar (Difco) plates. Fresh cultures were prepared for each experiment by inoculating bacteria onto new plates and incubating the plates at 37°C for 18 h. Several bacterial colonies were pooled and suspended in phosphate-buffered saline to a final concentration of approximately 10,000 CFU/ml. *P. aeruginosa* strain FRD2128(pJKG107), in which the LasA protease gene is carried on a plasmid under the control of the inducible *trc* promoter (15), served as the source for LasA protease. Bacteria were grown at 37°C with shaking in Luria broth supplemented with carbenicillin (100 μg/ml). For large-scale production of LasA protease, an overnight culture was diluted 1:100 with 3.2 liters of fresh medium and incubated for 12 h, at which time isopropyl-β-D-thiogalactopyranoside (1 mM) was added and the cells were incubated for an additional 12 h.

Purification of LasA protease. The cell-free culture medium obtained by centrifugation (6,000 × *g* for 20 min) of the LasA protease-producing *P. aeruginosa* cell culture was concentrated approximately eightfold with a Minitan concentration device (Millipore) and a set of four ultrafiltration membranes (cutoff, 10,000 Da; Millipore). The proteins in the resulting filtrate were concentrated further by ammonium sulfate (80% saturation) precipitation. The precipitate was dissolved in and dialyzed against 0.02 M Tris-HCl pH 8.0 (final volume, ~70 ml), and LasA protease was isolated from this fraction by chromatography on carboxymethyl cellulose, as described previously (23), with the exception that the salt gradient used to recover the enzyme from the column was from 0 to 1 M NaCl (instead of 0 to 0.6 M). The amount of LasA protease produced under these conditions was about 12 mg/liter, and the overall yield was between 50 and 60%. Fractions containing the purified enzyme (which was found to be homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie staining) were pooled and concentrated to about 2 mg/ml with a Diaflo concentration cell and a YM-10 membrane (cutoff, 10,000 Da; Amicon). The concentrated enzyme solution was dialyzed against 0.05 M HEPES–0.15 M NaCl (pH 7.8) (buffer A) and stored in aliquots at −80°C until use.

Antibiotics. Purified LasA protease, lysostaphin (Sigma, St. Louis, Mo.), and bovine serum albumin (BSA; Sigma) (control) were each diluted to a final concentration of 1 mg/ml in buffer A. Vancomycin (Merck Sharpe & Dohme, Lyon, France) was dissolved in sterile deionized water to a final concentration of 33 mg/ml, similar to that recommended for clinical use. A 50-mg/ml solution of cefazolin (Cefamezin) was obtained from the central pharmacy at the Sheba Medical Center, Tel-Hashomer, Israel. All antibiotic solutions were freshly prepared before use and kept at 4°C.

In vitro susceptibility. Inhibitory concentrations (ICs) were determined by the tube-broth dilution method in either cation-supplemented (for vancomycin and cefazolin) or regular (for LasA protease and lysostaphin) Mueller-Hinton broth (Becton Dickinson BBL) with a final inoculum of 10^5 CFU/ml. For determination of the ICs of lysostaphin and LasA protease, the broth was supplemented with 0.1% BSA (Sigma) to minimize adsorption of these enzymes to the walls of the test tubes. The IC_{50} was defined as the concentration that caused a 50% reduction in the absorbance at 600 nm of the bacterial cell suspension after 24 h of incubation at 37°C.

Induction of experimental *S. aureus* keratitis. New Zealand White rabbits (weight, 2.5 to 3.0 kg) were treated and maintained in accordance with the tenets of the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research, and the research was approved by the Tel Aviv University Institutional Review Board. All rabbits were anesthetized by intramuscular injection of xylazine (3 mg/kg of body weight) and ketamine hydrochloride (35 mg/kg). By observation through a stereoscopic microscope, the central corneal epithelium (diameter, 7.5 mm) was scraped with a Beever knife to allow maximal penetration of the enzymes applied. A total of 100 μl of a freshly prepared *S. aureus* cell suspension (approximately 1,000 organisms) was then injected intrastromally into the center of the cornea with a 30-gauge needle and a 1-ml tuberculin syringe.

Treatment. Following intrastromal injection of the bacterial cell suspension, the rabbits were randomly divided into treatment groups, as indicated below. Two therapeutic regimens were used. In the early-onset therapy schedule, infection was allowed to progress undisturbed for 4 h prior to the initiation of therapy and topical treatment was applied every 30 min during the first 5 h and then every hour for four additional hours. In the late-onset therapy schedule, infection was allowed to proceed for 10 h and topical treatment was applied every 30 min for 5 h. A single drop (45 μl containing 45 μg of LasA protease, lysostaphin, or BSA; 1.5 mg of vancomycin; and 2.25 mg of cefazolin) was applied at each time point, as specified above.

TABLE 1. IC_{50}s of the staphylolytic enzymes and antibiotics for *S. aureus*

Strain	IC_{50} (μg/ml)			
	LasA protease	Lysostaphin	Vancomycin	Cefazolin
MSSA	5	0.1	2	0.5
MRSA	2	0.1	2	200

Clinical evaluation. Approximately 45 min after administration of the last eye drop, all rabbits underwent biomicroscopic examination by a masked observer. Clinical parameters that were evaluated included conjunctival injection, chemosis, corneal infiltrate, corneal edema, fibrin in the anterior chamber, hypopyon formation, and iritis. Each parameter was graded on a scale of 0 to 4. The parameter grades were totaled to produce a single clinical score ranging from 0 (normal eye) to a theoretical maximum of 28 (9).

Bacterial quantification. One hour after the cessation of treatment, the rabbits were anesthetized and killed by intracardiac injection of pentobarbital (60 mg/ml). Uniform corneal buttons were removed aseptically with a 7.5-mm corneal trephine. The central corneal buttons were rinsed and homogenized in sterile phosphate-buffered saline (3 ml/cornea) with a Polytrone homogenizer. Aliquots of the corneal homogenates were serially diluted in the same buffer, plated in triplicate onto Muller-Hinton agar plates (100 μl per plate), and incubated for 24 h at 37°C to determine the number of CFU per cornea. The numbers of CFU were derived from dilutions that yielded 20 to 100 colonies per plate. Aliquots from homogenates of corneas that contained few or no organisms were plated undiluted. The lower limit of detection was 1 colony per plate, i.e., 30 CFU per cornea.

Statistical analysis. The numbers of CFU were tested for statistical significance by nonparametric analyses, Wilcoxon rank-sum tests, and analysis of variance (ANOVA) on the ranks. Evaluation of the clinical scores was performed by using ANOVA for comparison among groups. *P* values of 0.05 or smaller were considered significant. Before the analyses described above were carried out, variance component models were used to confirm that the data from several independent experiments could be analyzed at the individual-animal level.

RESULTS

In vitro susceptibilities of *S. aureus* strains. The IC_{50}s of LasA protease for the MSSA and MRSA strains used in this study were compared to those of lysostaphin, vancomycin, and cefazolin. Table 1 shows that the IC_{50} of LasA protease for the MSSA strain was 2.5-fold higher than that for the MRSA strain (5 and 2 μg/ml, respectively), indicating that the MRSA strain was more susceptible to LasA protease than its methicillin-sensitive counterpart. No difference was found between the susceptibilities of each of these strains to either lysostaphin or vancomycin as their respective IC_{50}s, 0.1 μg/ml for lysostaphin and 2 μg/ml for vancomycin, were found to be the same. The IC_{50}s of lysostaphin for both strains were lower than those of LasA protease by at least 1 order of magnitude, indicating that lysostaphin is more efficient than LasA protease in inhibiting the growth of *S. aureus* in vitro. The IC_{50}s (2 to 5 μg/ml) of vancomycin and LasA protease for both strains were comparable. The resistance of the MRSA strain to methicillin was confirmed by showing that the cefazolin IC_{50} for the strain (200 μg/ml) was 400 times higher than that for the MSSA strain (0.5 μg/ml).

Early-onset treatment of experimental keratitis. The efficacy of LasA protease in the treatment of MSSA- and MRSA-induced experimental keratitis was examined initially by using the early-onset therapy schedule. In experiments with the

TABLE 2. Bacterial numbers in corneas infected with either MSSA or MRSA after early initiation of therapy

MSSA				MRSA			
Expt no.	No. of CFU/cornea (10^4)[a]			Expt no.	No. of CFU/cornea (10^4)[a]		
	LasA	Lysostaphin	Control		LasA	Vancomycin	Control
I	0.09	0.27	160	I	<0.003	<0.003	330
	<0.003	<0.003	800		<0.003	<0.003	105
	<0.003	<0.003	600		<0.003	<0.003	370
	<0.003	<0.003	<0.003		<0.003	<0.003	12
II	84	<0.003	80	II	<0.003	<0.003	6
	<0.003	<0.003	1,800		<0.003	<0.003	108
	<0.003	<0.003	300		<0.003	<0.003	15
	<0.003	<0.003	1,600		<0.003	<0.003	6
				III	<0.003		156
					<0.003		14.7
					<0.003		96
					<0.003		<0.003
No. of eyes	8	8	8		12	8	12
Median[b]	0	0	450		0	0	55.5

[a] Values represent the results obtained for individual eyes in two to three independent experiments (Roman numerals) performed under identical conditions, with each experiment comprising four rabbits per treatment group.
[b] In calculating the median, numbers of CFU below $<0.003 \times 10^4$ were considered zero (sterile corneas).

MSSA strain, the effect of LasA protease was compared to that of lysostaphin, whereas in the case of the MRSA-infected corneas, the effectiveness of LasA protease was compared to that of vancomycin. Therefore, together with the control group (topical application of BSA), each set of experiments included three treatment groups. Table 2 shows that LasA protease was as effective as lysostaphin in eradicating the MSSA strain from the infected corneas. Examination of the numbers of CFU obtained for individual corneas for each of the treatment groups showed that LasA protease and lysostaphin both sterilized at least six of the eight infected corneas, and the numbers of CFU for the remaining eyes were reduced dramatically compared to those for most of the control eyes. Furthermore, the median numbers of CFU calculated for the LasA protease- and lysostaphin-treated eyes were 0, whereas the median number of CFU calculated for the control group was 4.5×10^6 CFU/cornea. LasA protease was even more effective in eradicating the MRSA strain, sterilizing all 12 eyes in three independent experiments; and it was as effective as vancomycin, which sterilized all eight eyes in this treatment group. Initial statistical analysis of the numbers of CFU obtained for individual eyes in each of several independent experiments (see Roman numerals in Table 2) showed that the variance between different experiments was negligible compared to the variance between rabbits within each experiment. Therefore, we concluded that a valid analysis of the data obtained for eyes receiving the same treatment in independent experiments could be conducted at the individual-animal level. Also, since the distribution of CFU in all of the treatment groups was highly nonsymmetric, with numerous values below the limit of detection ($<0.003 \times 10^4$; Table 2 [see also Table 4]), the analysis was performed on the ranks of the CFU counts rather than on the log scale of the actual data. The results of the statistical analysis confirmed the self-evident conclusions outlined above, clearly indicating that the numbers of CFU in the LasA protease-treated eyes were significantly lower than those in the eyes of the controls ($P < 0.001$). Using a nonparametric Wilcoxon test, we found no significant difference between the numbers of bacteria in the LasA protease- and lysostaphin-treated corneas ($P = 0.59$). Thus, despite the marked differences in their respective IC_{50}s (Table 1), LasA protease and lysostaphin were found to be as effective as each other when they were tested in vivo.

Table 3 shows that the average clinical score (3.8 ± 1.5) of the LasA protease-treated eyes in the MSSA keratitis model was lower than that obtained for the respective controls (6.6 ± 1.8). The same trend was observed in the MRSA keratitis model, in which the average clinical score for the LasA-treated eyes (2.3 ± 1.7) was far lower than that for the respective control eyes (4.3 ± 2.1). A statistical analysis performed on the sum of the individual values obtained for all of the LasA-treated eyes and that obtained for all of the control eyes, whether they were infected with MSSA or MRSA, demonstrated that the average clinical score for the LasA-treated eyes (2.93) was significantly lower than that for the control eyes (5.21; $P < 0.001$). The average score for the LasA protease-treated eyes in the MSSA keratitis model was, however, significantly higher than that for the eyes of the lysostaphin treatment group (3.8 and 1.9, respectively; $P = 0.004$) (Table 3), suggesting that either LasA protease was more irritative to the cornea than lysostaphin or it was less efficient than lysostaphin in eradicating the MSSA cells so that full elimination of the bacteria was attained at a later time point in the experiment.

Late-onset treatment of experimental keratitis. The late-onset therapy schedule provided a more extreme test of the efficacy of LasA protease, as it allowed a longer time for the bacteria to multiply freely in the cornea than was allowed in the early-onset therapy schedule. The experiments were performed with the same MSSA and MRSA strains used for early-onset therapy. Table 4 shows that despite the challenge

TABLE 3. Clinical scores after early initiation of therapy

MSSA				MRSA			
Expt	Total score[a]			Expt	Total score[a]		
	LasA	Lysostaphin	Control		LasA	Vancomycin	Control
I	2	2	5	I	2	2	7
	3	3	7		1	3	4
	6	2	7		1	2	3
	3.5	1.5	4		1	2	2
II	3	2	7.5	II	6	1	4
	4	2	6		4	1	6
	6	1.5	6		3	3	8
	3	1.5	10		0	2	3
				III	3		1
					1.5		5
					4		4
					1.5		ND[b]
No. of eyes	8	8	8		12	8	12
Mean	3.8	1.9	6.6		2.3	2	4.3
SD[c]	1.5	0.5	1.8		1.7	0.8	2.1

[a] Values represent the total scores obtained for individual eyes in either two or three independent experiments (Roman numerals) performed under identical conditions, with each experiment comprising four rabbits per treatment group.
[b] ND, not determined.
[c] SD, standard deviation.

of the late initiation of therapy, LasA protease remained highly effective in eradicating both the MSSA and the MRSA cells. As reflected by the median numbers of CFU calculated for eyes in each of the treatment groups, LasA protease reduced the number of viable bacteria in the corneas by 3 (MSSA) to 4 (MRSA) orders of magnitude compared to those for the controls (median values, 0.138×10^4 and 0.003×10^4 CFU/cornea, respectively, compared to 117×10^4 and 51×10^4 CFU/cornea in the respective controls). Strikingly, in experiments with the MRSA strain, LasA protease was even more effective in eradicating the bacteria than vancomycin (median numbers of CFU, 0.003×10^4 and 6.75×10^4, respectively; Table 4). A Wilcoxon test performed on the ranks of the actual numbers of CFU confirmed that the numbers of CFU obtained for the LasA protease treatment group were significantly lower than those obtained for both the control ($P = 0.001$) and the vancomycin ($P = 0.02$) treatment groups.

Table 5 shows that despite the late initiation of therapy, the clinical scores for eyes treated with LasA protease, whether the eyes were infected with the MSSA strain or the MRSA strain (average scores, 2.5 ± 2.1 and 2.9 ± 0.99, respectively), were lower than those for the control eyes (4.1 ± 2.4 and 4.4 ± 1.8,

TABLE 4. Bacterial numbers in corneas infected with either MSSA or MRSA after late initiation of therapy

MSSA			MRSA			
Expt	No. of CFU/cornea (10^4)[a]		Expt	No. of CFU/cornea (10^4)[a]		
	LasA	Control		LasA	Vancomycin	Control
I	190	1,500	I	0.75	10	67
	30	960		0.006	1.4	54
	0.21	54		0.008	<0.003	<0.003
	0.007	2.3		7.2	28	78
	0.05	180				
	<0.003	<0.003				
II	0.07	33	II	<0.003	0.01	49
	0.01	190		<0.003	45	7.5
	36	33		<0.003	3.5	0.2
	80	576		<0.003	14.5	72
	94	0.86				
	0.01	290				
No. of eyes	12	12		8	8	8
Median[b]	0.138	117		0.003	6.75	51.5

[a] Values represent the results obtained for individual eyes in two independent experiments (Roman numerals), each of which was performed under identical conditions, with each experiment comprising either four (MRSA) or six (MSSA) rabbits per treatment group.
[b] In calculating the median, numbers of CFU below $<0.003 \times 10^4$ were considered zero (sterile corneas).

TABLE 5. Clinical scores after late initiation of therapy

MSSA			MRSA			
Expt	Total score[a]		Expt	Total score[a]		
	LasA	Control		LasA	Vancomycin	Control
I	5	1	I	3	3	3
	0	2		2	2	1
	4	2.5		2	2	3
	1	1		3	3	6
	0	5				
	2	6				
II	3.5	4	II	5	2	6
	1	5		3	6	6
	3	5		3	2.5	5
	7	10		2	4.5	5
	2	4				
	2	3.5				
No. of eyes	12	12		8	8	8
Mean	2.5	4.1		2.9	3.1	4.4
SD[b]	2.1	2.4		0.99	1.4	1.8

[a] Values represent the total scores obtained for individual eyes in two independent experiments (Roman numerals) performed under identical conditions, with each experiment comprising either four (MRSA) or six (MSSA) rabbits per treatment group.
[b] SD, standard deviation.

respectively). Also evident from Table 5 is that in the MRSA model, the average clinical score for the LasA protease-treated eyes (2.9 ± 0.99) was practically the same as that for the vancomycin-treated eyes (3.1 ± 1.4), indicating that the degree of irritation caused by both agents was comparable. Statistical analysis (ANOVA for comparison among groups) performed with the combined clinical scores for all of the LasA-treated eyes and those for the respective control eyes confirmed that the differences between the scores for the two groups (2.65 and 4.18 for the LasA protease-treated eyes and control eyes, respectively) were significant ($P = 0.01$).

Comparison of early- and late-onset therapy protocols. An additional statistical analysis was performed to compare the efficacy of LasA protease in the early-onset therapy schedule to its efficacy in the late-onset therapy schedule. Since the protective effect of LasA protease against both the MSSA and the MRSA strains was consistent, the ranks of the numbers of CFU from experiments with both strains were analyzed jointly. The difference between the average CFU ranks for the LasA protease-treated eyes and the control eyes was larger for the early-treatment protocol (average ranks, 17.4 for LasA protease-treated eyes and 57.7 for the control eyes; $P < 0.001$) than for the late-treatment protocol (average ranks, 34.5 for the LasA protease-treated eyes and 52.4 for the control eyes; $P = 0.001$). This emphasized the higher potency of LasA protease than that of the control treatment in the early-treatment protocol compared to its more modest (although significant) effect in the late-treatment protocol. In addition, the average clinical scores for the LasA protease-treated eyes and those for the respective controls after early and late treatments were compared. Again, data from experiments with MSSA- and MRSA-infected eyes were analyzed jointly. The differences between the average scores for the LasA treatment group and those for the control group were larger for the early initiation of treatment (2.9 and 5.2, respectively; $P < 0.001$) than for the late initiation of treatment (2.7 and 4.2, respectively; $P = 0.01$). Thus, the clinical improvement of the LasA-treated eyes was more moderate in the late-treatment protocol than in the early-treatment protocol.

DISCUSSION

The treatment of serious infections caused by MRSA requires intensive administration of bactericidal antibiotics. Since MRSA strains are resistant to most β-lactams, glycopeptides such as vancomycin are considered the treatment of choice. Despite the universal susceptibility of *S. aureus* to vancomycin, clinical failures of vancomycin therapy for MRSA infections have recently become more common due to the shifting trends in the susceptibilities of certain *S. aureus* strains (14, 25, 32, 34, 36). Of special concern in this regard is the recent emergence of MRSA strains with intermediate resistance to glycopeptides, known as glycopeptide-intermediate or vancomycin-intermediate strains (12, 34, 36). Infections with such strains cannot be effectively treated with vancomycin alone (28), which highlights the urgent need for new broad-range and potent antistaphylococcal therapies.

The discovery that LasA protease is a potent staphylolytic protease (20) suggested that it might lend itself to the treatment of *S. aureus* infections. The ability of LasA protease to eradicate *S. aureus* in vivo has not been documented to date. Using an experimental model of *S. aureus* keratitis in rabbit eyes, we showed for the first time that LasA protease can kill *S. aureus* cells in vivo and established its potential as a tool in the management of *S. aureus* keratitis. Since the efficacy of lysostaphin has been evaluated in a similar model of *S. aureus* keratitis (9, 11), it was of interest to compare the efficacy of LasA protease with that of lysostaphin, an issue that we have addressed in the first set of experiments. Using the early-onset therapy protocol and the MSSA strain as the target organism,

we found that even though the IC_{50} of LasA protease for this strain was considerably higher than that of lysostaphin (Table 1), in an in vivo setting, LasA protease was as effective as lysostaphin, with both agents sterilizing essentially all of the treated eyes (Table 2). The apparent discrepancy between the in vitro and in vivo activities of LasA protease and lysostaphin may be explained by the fact that lysostaphin (but not LasA protease) possesses a unique C-terminal domain that binds to the cell walls of *S. aureus* (4). Binding of lysostaphin to its target bacteria enhances its potency during planktonic growth, accounting for its extremely low IC_{50} (Table 1). It is possible that at lower doses the enhanced potency of lysostaphin within the cornea also becomes important and that although the bacteria were eradicated equally at the time point studied, bacterial clearance with lysostaphin proceeded faster than that with LasA protease. This may also explain the differences in the clinical scores observed with each drug.

The ability of LasA protease to eradicate the MSSA and MRSA strains was compared by using both the early- and the late-onset therapy schedules. The results showed that although the ability of LasA protease to eradicate the bacteria when it was applied late after infection was lower than that when it was applied early after infection, it offered a highly effective therapy for experimental keratitis caused by both the MSSA and the MRSA strains. The reduced effectiveness of LasA protease at the late-onset therapy is in accordance with previous observations of Dajcs et al. (9, 11), who showed that when lysostaphin was applied late after infection, it did not sterilize the infected corneas, even though it was given at a concentration of 2.8 mg/ml, almost threefold higher than the 1 mg/ml used here. Thus, with both enzymes, early treatment seems to be an advantage.

The higher sensitivity of the MRSA strain to LasA protease, which became apparent in the late-treatment protocol (Table 4), is in good correlation with the lower IC_{50} determined for this strain in vitro (Table 1) and is reflected by the relatively low clinical score (2.3; Table 3) determined for this strain in the early-treatment protocol. Importantly, the efficacy of LasA protease against the MRSA strain was either the same as that of vancomycin (early initiation of therapy; Table 2) or higher than that of vancomycin (late initiation of therapy; Table 4), while the clinical scores for eyes treated with either LasA protease or vancomycin were essentially the same (Tables 3 and 5). The superiority of LasA protease over vancomycin when the drugs were given late after infection suggests that, unlike vancomycin, which has limited activity against resting cells (6, 9), LasA protease can lyse bacterial cells at their stationary phase of growth, when cell proliferation is minimal. Tobramycin is the only other antibacterial agent known to maintain its effectiveness in both the early and the late phases of experimental *Staphylococcus* keratitis. However, tobramycin is normally not effective against MRSA strains (7).

In conclusion, LasA protease may offer an effective antibacterial therapy that can be used to control severe MSSA or MRSA keratitis. To further establish its therapeutic potential, studies that evaluate its activity against a range of MRSA and vancomycin-intermediate *S. aureus* strains are in order. Studies that examine the efficacy of LasA protease as an adjunctive treatment in combination with other antibiotics are also warranted.

ACKNOWLEDGMENTS

This study was supported by grants from the Maratier and Schreiber Foundations of the Tel-Aviv University Sackler Faculty of Medicine (to I.S.B. and E.K.), the Tel-Aviv University Foundation for Basic Research (to E.K.), Public Health Service grant AI 26187 from the National Institute of Allergy and Infectious Diseases (to D.E.O.), and in part, by Veterans Administration Medical Research Funds (to D.E.O.).

We thank David Steinberg for assistance with statistical analyses and Nathan Keller for providing the MRSA strain.

REFERENCES

1. **Alexandrakis, G., E. C. Alfonso, and D. Miller.** 2000. Shifting trends in bacterial keratitis in south Florida and emerging resistance to fluoroquinolones. Ophthalmology **107:**1497–1502.
2. **Alionte, L. G., B. M. Cannon, C. D. White, R. J. Caballero, R. J. O'Callaghan, and J. A. Hobden.** 2001. *Pseudomonas aeruginosa* LasA protease and corneal infections. Curr. Eye Res. **22:**266–271.
3. **Asbell, P., and S. Stenson.** 1982. Ulcerative keratitis. Survey of thirty years' laboratory experience. Arch. Ophthalmol. **100:**77–80.
4. **Baba, T., and O. Schneewind.** 1996. Target cell specificity of a bacteriocin molecule: a C-terminal signal directs lysostaphin to the cell wall of *Staphylococcus aureus.* EMBO J. **15:**4789–4797.
5. **Brito, N., M. A. Falcon, A. Carnicero, A. M. Gutierrez-Navarro, and T. B. Mansito.** 1989. Purification and peptidase activity of a bacteriolytic extracellular enzyme from *Pseudomonas aeruginosa.* Res. Microbiol. **140:**125–137.
6. **Callegan, M. C., J. M. Hill, M. S. Insler, J. A. Hobden, and R. J. O'Callaghan.** 1992. Methicillin-resistant *Staphylococcus aureus* keratitis in the rabbit: therapy with ciprofloxacin, vancomycin and cefazolin. Curr. Eye. Res. **11:**1111–1119.
7. **Callegan, M. C., R. J. O'Callaghan, and J. M. Hill.** 1994. Pharmacokinetic considerations in the treatment of bacterial keratitis. Clin. Pharmacokinet. **27:**129–149.
8. **Climo, M. W., R. L. Patron, B. P. Goldstein, and G. L. Archer.** 1998. Lysostaphin treatment of experimental methicillin-resistant *Staphylococcus aureus* aortic valve endocarditis. Antimicrob. Agents Chemother. **42:**1355–1360.
9. **Dajcs, J. J., E. B. Hume, J. M. Moreau, A. R. Caballero, B. M. Cannon, and R. J. O'Callaghan.** 2000. Lysostaphin treatment of methicillin-resistant *Staphylococcus aureus* keratitis in the rabbit. Investig. Ophthalmol. Vis. Sci. **41:**1432–1437.
10. **Dajcs, J. J., B. A. Thibodeaux, E. B. Hume, X. Zheng, G. D. Sloop, and R. J. O'Callaghan.** 2001. Lysostaphin is effective in treating methicillin-resistant *Staphylococcus aureus* endophthalmitis in the rabbit. Curr. Eye Res. **22:**451–457.
11. **Dajcs, J. J., B. A. Thibodeaux, D. O. Girgis, M. D. Shaffer, S. M. Delvisco, and R. J. O'Callaghan.** 2002. Immunity to lysostaphin and its therapeutic value for ocular MRSA infections in the rabbit. Investig. Ophthalmol. Vis. Sci. **43:**3712–3716.
12. **Daum, R. S., and J. B. Seal.** 2001. Evolving antimicrobial chemotherapy for *Staphylococcus aureus* infections: our backs to the wall. Crit. Care Med. **29**(Suppl.)**:**N92–N96.
13. **Dixon, R. E., J. S. Goodman, and M. G. Koenig.** 1968. Lysostaphin; an enzymatic approach to staphylococcal disease. III. Combined lysostaphin-methicillin in therapy of established staphylococcal abscesses in mice. Yale J. Biol. Med. **41:**62–68.
14. **Goldstein, M. H., R. P. Kowalski, and Y. J. Gordon.** 1999. Emerging fluoroquinolone resistance in bacterial keratitis: a 5-year review. Ophthalmology **106:**1313–1318.
15. **Gustin, J. K., E. Kessler, and D. E. Ohman.** 1996. A substitution at His-120 in the LasA protease of *Pseudomonas aeruginosa* blocks enzymatic activity without affecting propeptide processing or extracellular secretion. J. Bacteriol. **178:**6608–6617.
16. **Harris, R. L., A. W. Nunnery, and H. D. Riley.** 1967. Effects of lysostaphin on staphylococcal carriage in infants and children. Antimicrob. Agents Chemother. **7:**110–112.
17. **Harrison, E. F., and C. B. Cropp.** 1967. Therapeutic activity of lysostaphin in experimental staphylococcal infections. Can. J. Microbiol. **13:**93–97.
18. **Hobden, J. A.** 2002. *Pseudomonas aeruginosa* proteases and corneal virulence. DNA Cell Biol. **21:**391–396.
19. **Kessler, E.** 1995. β-Lytic endopeptidases. Methods Enzymol. **248:**740–756.
20. **Kessler, E., M. Safrin, J. C. Olson, and D. E. Ohman.** 1993. Secreted LasA protease of *Pseudomonas aeruginosa* is a staphylolytic protease. J. Biol. Chem. **268:**7503–7508.
21. **Kessler, E., and D. E. Ohman.** 1998. Staphylolysin, p. 1476–1478. *In* A. J. Barrett, N. D. Rawlings, and J. F. Woessner (ed.), Handbook of proteolytic enzymes, 1st ed. Academic Press, London, United Kingdom.
22. **Kessler, E., M. Safrin, W. R. Abrams, J. Rosenbloom, and D. E. Ohman.** 1997. Inhibitors and specificity of *Pseudomonas aeruginosa* LasA protease. J. Biol. Chem. **272:**9884–9889.

23. **Kessler, E., M. Safrin, J. K. Gustin, and D. E. Ohman.** 1998. Elastase and the LasA protease of *Pseudomonas aeruginosa* are secreted with their propeptides. J. Biol. Chem. **273:**30225–30231.
24. **Liesegang, T. J.** 1998. Bacterial and fungal keratitis, p. 159–219. *In* H. E. Kaufman, B. A. Baron, and M. B. McDonald (ed.), The cornea, 2nd ed. Butterworth-Heinemann, Boston, Mass.
25. **Maffett, M., and D. M. O'Day.** 1993. Ciprofloxacin-resistant bacterial keratitis. Am. J. Ophthalmol. **115:**545–546.
26. **Mansito, T. B., M. A. Falcon, J. Moreno, A. Carnicero, and A. M. Gutierrez-Navarro.** 1987. Effects of staphylolytic enzymes from *Pseudomonas aeruginosa* on the growth and ultrastructure of *Staphylococcus aureus.* Microbios **49:**55–64.
27. **O'Brien T. P.** 1997. Bacterial keratitis, p. 1139–1189. *In* J. H. Krachmer, M. J. Mannis, and E. J. Holland (ed.) Cornea and external disease: clinical diagnosis and management. Mosby-Year Book, St. Louis, Mo.
28. **Patron, R. L., M. W. Climo, B. P. Goldstein, and G. L. Archer.** 1999. Lysostaphin treatment of experimental aortic valve endocarditis caused by a *Staphylococcus aureus* isolate with reduced susceptibility to vancomycin. Antimicrob. Agents Chemother. **43:**1754–1755.
29. **Perestelo, F. R., M. T. Blanco, A. M. Gutierre-Navarro, and M. A. Falcon.** 1985. Growth inhibition of *Staphylococcus aureus* by a staphylolytic enzyme from *Pseudomonas aeruginosa.* Microbios Lett. **30:**85–94.
30. **Preston, M. J., P. C. Seed, D. S. Toder, B. H. Iglewski, D. E. Ohman, J. K. Gustin, J. B. Goldberg, and G. B. Pier.** 1997. Contribution of proteases and LasR to the virulence of *Pseudomonas aeruginosa* during corneal infections. Infect. Immun. **65:**3086–3090.
31. **Quickel, K. E., R. Selden, J. R. Caldwell, N. F. Nora, and W. Schaffner.** 1971. Efficacy and safety of topical lysostaphin treatment of persistent nasal carriage of *Staphylococcus aureus.* Appl. Microbiol. **22:**446–450.
32. **Schaefer, F., O. Bruttin, L. Zografos, and Y. Guex-Crosier.** 2001. Bacterial keratitis: a prospective clinical and microbiological study. Br. J. Ophthalmol. **85:**842–847.
33. **Schindler, C. A., and V. T. Schuhardt.** 1964. Lysostaphin: A new bacteriolytic agent for *Staphylococcus.* Proc. Natl. Acad. Sci. USA **51:**414–421.
34. **Srinivasan, A., J. D. Dick, and T. M. Perl.** 2002. Vancomycin resistance in staphylococci. Clin. Microbiol. Rev. **15:**430–438.
35. **Vessillier, S., F. Delorme, J. Bernillon, J. Saulnier, and J. Wallach.** 2001. Hydrolysis of glycine-containing elastin pentapeptides by LasA protease, a metalloelastase from *Pseudomonas aeruginosa.* Eur. J. Biochem. **268:**1049–1057.
36. **Walsh, T. R., and R. A. Howe.** 2002. The prevalence and mechanisms of vancomycin resistance in *Staphylococcus aureus.* Annu. Rev. Microbiol. **56:** 657–675.
37. **White, C. D., L. G. Alionte, B. M. Cannon, A. R. Caballero, R. J. O'Callaghan, and J. A. Hobden.** 2001. Corneal virulence of LasA protease-deficient *Pseudomonas aeruginosa* PAO1. Cornea **20:**643–646.
38. **Zygmunt, W., and P. A. Tavormina.** 1972. Lysostaphin: model for a specific enzymatic approach to infectious disease. Prog. Drug Res. **16:**309–333.