

Inhibitory Effect of a Complementary Peptide on Ulceration in the Alkali-Injured Rabbit Cornea

Jeffrey L. Haddox,¹ Roswell R. Pfister,¹ Charnell I. Sommers,¹
J. Edward Blalock,² and Matteo Villain²

PURPOSE. Two tripeptide chemoattractants, acetyl-proline-glycine-proline (Ac-PGP) and methyl-proline-glycine-proline (Me-PGP), are the primary triggers for early neutrophil invasion into the alkali-injured cornea. In the present study the effectiveness of a complementary peptide designed to inhibit the PGP chemoattractants (arginine-threonine-arginine [RTR] tetrameric peptide) and an apo A-1 mimicking peptide (5F) was investigated in the alkali-injured rabbit eye.

METHODS. (L)-RTR tetramer, (D)-RTR tetramer, and 5F were tested in vitro for their effects on neutrophil polarization. Synthetic 5F was also tested in vitro for its effect on the neutrophil respiratory burst. In the alkali-injured rabbit eye model, the right corneas of 48 rabbits were exposed to 1 N NaOH for 35 seconds. Sixteen animals were randomly assigned to each of three groups: phosphate-buffered saline (PBS) control; 800 μ M RTR (dextrorotatory) tetramer in PBS alternating each hour with 1.5 mM RTR (levorotatory) tetramer in PBS; and 12 μ M 5F in PBS. One topical drop of each substance was administered hourly (14 times per day) for 33 days. The experiment was continued until day 42 with no additional drops administered.

RESULTS. (L)-RTR tetramer and (D)-RTR tetramer inhibited neutrophil polarization activated by the PGP chemoattractants in vitro. Synthetic 5F did not inhibit neutrophil polarization in the presence of Ac-PGP or the respiratory burst of neutrophils in the presence of a metabolic stimulant derived from alkali-degraded corneas. During the entire animal experiment, statistically fewer ulcers occurred in the RTR tetramer group than in the PBS control group (43.8% vs. 87.5%, $P = 0.0046$). The frequency of ulceration in the 5F group (68.8%) was not significantly different from the PBS control group.

CONCLUSIONS. The reduction in the frequency of corneal ulceration by the RTR tetramer possibly resulted from its complementary binding to Ac-PGP and Me-PGP in the cornea shortly after alkali injury, leading to a reduction in the early and late infiltration of neutrophils. RTR tetramer appears to hold enough promise to warrant additional study as a therapeutic drug for the alkali-injured eye. (*Invest Ophthalmol Vis Sci.* 2001;42:2769-2775)

Alkali injury of the eye provokes a sterile, acute, inflammatory reaction, frequently leading to corneal ulceration and perforation.¹⁻³ This inflammatory cellular response is largely composed of polymorphonuclear leukocytes (PMNs). A number of inflammatory mediators have been found in the alkali-injured eye, but on the basis of prior investigations acetyl-proline-glycine-proline (Ac-PGP) and methyl-proline-glycine-proline (Me-PGP) were identified as the primary triggers for early neutrophil invasion into the alkali-injured cornea.⁴⁻⁶ These chemotactic mediators are unique in that they result from direct alkaline hydrolysis of corneal proteins.

Recognition that acetylated and methylated PGP were important mediators in this disease opened a window of therapeutic opportunity. One approach to the development of a compound inhibitory for this chemoattractant is the sense-antisense, or molecular recognition, theory.⁷ This concept posits that protein molecules recognize one another in a genetically defined manner. Blalock and Smith⁸ proposed a novel approach to molecular recognition that succeeded in predicting protein interactions with high accuracy. This method is based on the development of complementary peptides specified by ligand antisense RNA. It has been useful in designing interactive peptides, isolating receptors, and producing anti-receptor and anti-idiotypic antibodies.^{9,10} Using this approach, the arginine-threonine-arginine (RTR) amino acid sequence was incorporated into a tetrameric peptide designed to bind to the complementary PGP sequence. In a recent in vitro study, the RTR tetramer was shown to be a potent inhibitor of neutrophils activated by the Ac-PGP chemoattractant.¹¹

Exclusion of neutrophils from the alkali-injured cornea is the likely key to decreasing or eliminating corneal ulceration. Inhibition of the PGP chemoattractants by the RTR tetrameric peptide in an alkali-injured eye may reduce the first neutrophilic influx. The purpose of this study was to investigate the effectiveness of this RTR tetrameric peptide in vivo. In a second experimental group, a synthetic peptide (5F) was tested, because it mimics the activity of apo A-1, which is known to inhibit the respiratory burst and degranulation of neutrophils, but not chemotaxis.¹² The present study represents the first use of an antisense peptide as a therapeutic agent to treat eye disease by directly binding to, and thus inhibiting, inflammatory mediators.

MATERIALS AND METHODS

Materials

Hanks' balanced salt solution (HBSS) was purchased from Gibco Laboratories (Chagrin Falls, OH); calcium chloride, magnesium chloride, sodium chloride, glutaraldehyde, sodium hydroxide, and a nonionic synthetic polymer of sucrose (Ficoll; Type 400) from Sigma Chemical Co. (St. Louis, MO); sodium phosphate monobasic and sodium phosphate dibasic from Fisher Scientific (Fairlawn, NJ); diatrizoate meglumine and diatrizoate sodium (Hypaque-76) from Winthrop Laboratories (New York, NY); solvents for peptide synthesis from Fisher Scientific Products (West Chester, PA); the reagents from Perseptive Biosystems (Framingham, MA); Fmoc-d-Arg(Pbf)-OH and Fmoc-d-Thr(tBu)-OH from Chem-Impex (Wood Dale, IL); zymosan from Sigma

From the ¹Eye Research Laboratories, Sight Savers of Alabama, Birmingham; and the ²Department of Physiology and Biophysics, the University of Alabama at Birmingham.

Supported by National Eye Institute Grant EY04716 (JLH, RRP, CIS) and National Institutes of Health Grant MH52527 (JEB, MV). Mass spectrometry was performed at the University of Alabama at Birmingham Comprehensive Cancer Center Mass Spectrometry Shared Facility, which is supported in part by National Cancer Institute Core Research Support Grant P30CA13148.

Submitted for publication December 12, 2000; revised May 24, 2001; accepted June 27, 2001.

Commercial relationships policy: P.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Jeffrey L. Haddox, Sight Savers of Alabama, Eye Research Laboratories, 500 Robert Jemison Road, Birmingham, AL 35209. sightsavers@mindspring.com

Chemical Co.; and frozen bovine eyes from Pel-Freez Biologicals (Rogers, AR).

Peptide Synthesis

The RTR tetrameric peptide (((H2N-Arg-Thr-Arg-Gly-Gly)2-Lys)2-Lys-Ala-CONH2), containing levorotatory (L) RTR sequences, was purchased from Lipal Biochemicals (Gundetswil, Switzerland). The RTR tetrameric peptide (((H2N-d-Arg-d-Thr-d-Arg-Gly-Gly)2-Lys)2-Lys-Ala-CONH2), containing dextrorotatory (D) sequences, was synthesized manually in the Department of Physiology and Biophysics, University of Alabama at Birmingham, using stepwise solid-phase peptide synthesis, starting with 3g of the Fmoc-Pal-Peg-PS resin with an initial substitution of 0.2 mmol/g resin. Dimethylformamide (DMF) was used as the solvent for the coupling steps and the washing steps, whereas Fmoc deprotection was achieved with 1% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)-2% piperidine in DMF. Monitoring of the coupling and deprotection steps was conducted using the test by Kaiser et al.¹³ All amino acids were doubly coupled for 1 hour, using as activating reagents 1-hydroxy-7-azabenzotriazole (HOAt) for the *O*-pentafluorophenyl ester amino acid and 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetra-methyluronium hexafluorophosphate/diisopropylethylamine (HATU/DIPEA) for the free acids. An excess of 5 equivalents of amino acid over the resin substitution was used for alanine and the first lysine, 10 equivalents for the second lysine, and 20 equivalents for the following amino acids. The peptide was cleaved from the resins and purified.

For synthesis of Ac-PGP, the dipeptide *t*-Boc-PG was coupled to Pro-Merrifield resin using the dicyclohexylcarbodiimide/1-hydroxybenzotriazole procedure. After removal of the N-terminal protection and acetylation using acetic anhydride, the peptide was cleaved from the resin using anhydrous hydrofluoric acid and purified. Quantitative amino acid analysis was performed to show the correct ratio of amino acids and to determine the peptide content for calculation of the final concentration.

Me-PGP was purchased from Peninsula Laboratories (Belmont, CA). Synthetic 5F (acetyl-(AspTrpLeuLysAlaPheTyrAspLysValPheGluLysPheLysGluPhePhe)-NH₂) was synthesized as previously reported.¹⁴

Purification of synthetic peptides was achieved by preparative reversed-phase high-performance liquid chromatography using a C-18 column. All synthetic peptides were identified by mass spectroscopy.

In Vitro Studies

Preparation of Mediators and Inhibitors. Synthetic peptides were dissolved in HBSS (pH 7.3). When necessary, the osmolality was adjusted between 280 and 320 mOsm/kg by adding a small amount of distilled water. Zymosan was opsonized by incubating in human serum (10 mg/ml) for 30 minutes at 37°C, centrifuged, and rinsed in HBSS three times and resuspended in HBSS (10 mg/ml).

The respiratory burst stimulant from alkali-degraded corneas was isolated from whole bovine corneas. Corneas were excised from frozen eyes and alkali treated. The ratio of corneal dry weight to final volume of 1 N NaOH was 1:12 (wt/vol). The final volume and concentration of alkali included the water content of each cornea (456 μ l), based on preliminary studies of the difference between dry (83 mg) and wet (539 mg) weights. The following method was used: 100 corneas, containing 45.6 ml water, were added to 45.6 ml 2 N NaOH and mixed. The sample was then incubated at 35°C for 24 hours. The sample was neutralized by titration with 1 N HCl to pH 7.4 and 0.05% sodium azide was added. The crude suspension was centrifuged at 15,000g for 15 minutes and the supernatant fraction collected.

The supernatant fraction was then placed in a 30,000-MWt cutoff system (Centriprep; Amicon, Beverly, MA) and centrifuged at 1500g for 30 minutes. The ultraconcentrate was titrated with acetic acid to pH 5.0 and centrifuged at 15,000g for 5 minutes. The resultant pellet was extracted with 100% acetonitrile, sonicated for 30 minutes, and centrifuged at 15,000g for 5 minutes and the supernatant fraction evaporated to dryness. The residue from the acetonitrile extract was resuspended in 1 ml of a mixture of distilled

water and HBSS. The osmolality was adjusted to between 270 and 310 mOsm and the pH to 7.3. A 1:50 dilution of this sample was used as the respiratory burst stimulant from alkali-degraded corneas to measure the PMN respiratory burst.

Neutrophil Isolation. These experiments followed the tenets of the Declaration of Helsinki and were approved by the Human Research Committee at Brookwood Medical Center (Birmingham, AL). All donors signed written consent forms that explained the nature and possible consequences of the study. Blood was collected from one donor each day by venipuncture. Using the technique of Ferrante and Thong,¹⁵ neutrophils were isolated from fresh heparinized human blood by centrifugation with a single-density gradient (optical density, 1.114; Hypaque, Winthrop Laboratories; Ficoll, Sigma Chemical Co.), in accordance with a previous paper.¹⁶ Isolated neutrophils (96%–99% viability) were resuspended in HBSS, containing 15 mM phosphate buffer, 500 μ M Ca²⁺, and 500 μ M Mg²⁺, at room temperature and gently agitated on a shaker. The purity of this cell suspension was more than 85% neutrophils and less than 5% mononuclear cells and platelets, with the remaining percentage consisting of red blood cells.

Purified neutrophils were used in the polarization and respiratory burst assays. All incubation mixtures were maintained between an osmolality of 280 to 320 mOsm and a pH range of 7.2 to 7.6. Cell viability for the polarization and respiratory burst assay samples was within control limits (<5% cell death) as measured by lactic dehydrogenase activity^{17,18} and trypan blue staining. The Student's *t*-test was used to analyze data from the neutrophil polarization and respiratory burst experiments.

Polarization Assay. This *in vitro* assay was used, before the animal experiment, to measure the effect of inhibitors ([D]-RTR tetramer, [L]-RTR tetramer, and 5F) on neutrophil polarization activated by Ac-PGP or Me-PGP. The polarization assay was performed in a masked fashion. The polarization index is a measure of the frequency and degree of cellular shape change after exposure to a chemoattractant.¹⁹ Briefly, preincubated neutrophils (2×10^5) were mixed with preincubated chemoattractants and/or inhibitors in a 100- μ l reaction chamber at 37°C for 5 minutes. At the end of the incubation period, an aliquot was collected and mixed with an equal volume of 4% glutaraldehyde for microscopic observation. Neutrophils in each sample were observed microscopically and assigned scores of 0 (resting, spherical cell with a smooth membrane), 1 (activated, irregular cell with uneven membranes), or 2 (polarized, cell length more than two times width). Scores of 100 neutrophils for each sample were added and corrected by subtracting negative control scores (neutrophils in HBSS only), producing a polarization index.

Respiratory Burst Assay. The respiratory burst assay was used to test the effectiveness of the 5F inhibitor on activation of the neutrophil respiratory burst before the alkali-injured rabbit eye experiment. This *in vitro* assay was performed in a masked fashion. A luminometer (Flyte 400; Cardinal Associates, Santa Fe, NM) was used to measure light (amplified by luminol and calibrated in relative light units) emitted by the decay of oxygen radicals.²⁰ These radicals are released during the PMN respiratory burst when triggered by opsonized zymosan or the respiratory burst stimulant from alkali-degraded corneas. The optimal doses, 600 ng/ml opsonized zymosan and a 1:50 dilution of the respiratory burst stimulant from alkali-degraded corneas, were chosen from preliminary dose-response studies. Briefly, 5×10^6 PMNs were suspended in HBSS, containing luminol (0.2 mg/ml) and dimethyl sulfoxide (DMSO; 0.0025%), and exposed to the 5F inhibitor in the presence of opsonized zymosan or the respiratory burst stimulant from alkali-degraded corneas. The samples were incubated in a luminometer reaction chamber (total volume, 250 μ l) at 35°C for 10 minutes.

In Vivo Study

Preparation of Dropping Solutions. (L)-RTR tetramer, (D)-RTR tetramer, and 5F were dissolved in physiologic phosphate-buffered saline (PBS) at pH 7.3 and 295–310 mOsm. Constituents of the PBS solution were as follows: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂

TABLE 1. Inhibition of Ac-PGP-Induced Neutrophil Polarization

Complementary Peptide	100 nM	1 μ M	10 μ M	ID ₅₀	P
(L)-RTR tetramer	21% \pm 15.1% (<i>n</i> = 2)	75% \pm 4.8% (<i>n</i> = 12)	94% \pm 2.5% (<i>n</i> = 5)	580 nM	<0.001
(D)-RTR tetramer	37% \pm 13.2% (<i>n</i> = 7)	65% \pm 10.6% (<i>n</i> = 6)	92% \pm 2.4% (<i>n</i> = 6)	520 nM	<0.001

Untreated neutrophils (negative control) produced a polarization response of 8% \pm 0.6% (*n* = 47). Neutrophils activated with 800 μ M Ac-PGP (positive control) produced a polarization response of 57% \pm 1.9% (*n* = 49). This concentration of the PGP chemoattractant was selected from the linear portion of the dose-response curve, yielding approximately 50% polarization after subtraction of the negative control values. Percent inhibition (mean \pm SEM) of Ac-PGP-induced neutrophil polarization at each concentration of both RTR tetramers was calculated after subtraction of the negative control from all values. Each ID₅₀ was interpolated from the RTR tetramer dose-response curve as the concentration required to produce 50% inhibition of the positive control. Student's *t*-test was used to analyze the statistical difference between the polarization response produced by the highest concentration of each RTR tetramer in the presence of 800 μ M Ac-PGP and the polarization response produced by the positive control (800 μ M Ac-PGP-activated neutrophils). ID₅₀, 50% inhibitory dose.

HPO₄ and 1.8 mM KH₂PO₄. The concentrations of both RTR tetramers were dictated by the limited quantities of synthetic RTR tetramer available. The highest achievable concentration of synthetic 5F (12 μ M) was dictated by its solubility in the PBS control vehicle.

Alkali Injury Model. Animals were maintained and treated in full compliance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. Forty-eight New Zealand Dutch strain albino rabbits (Myrtle's Rabbitry, Thompson Station, TN) weighing 2.0 to 2.5 kg were anesthetized with ketamine HCl (12 mg/kg) and xylazine (7.5 mg/kg). Two drops of topical proparacaine (Allergan, Hormigueros, Puerto Rico) were placed in the right eye of each rabbit. After ocular proptosis, a 12-mm plastic well was centered on the cornea, and 0.4 ml 1 N NaOH was instilled into the well and left for a period of 35 seconds. The alkali was aspirated and the well irrigated with 10 ml physiological saline. Erythromycin ointment (0.5%) was applied two times a day and study medications administered by the technique of Fraunfelder.²¹ Double-masked examinations (slit lamp and dissecting microscopy) were conducted on Monday, Wednesday, and Friday with photographs obtained on Wednesday. Evaluation was made for the presence and size of epithelial defects, corneal ulceration, perforation, and vascularization, using fluorescein where indicated. The determination of the severity of ulceration was based on the presence of ulceration into the anterior third, middle third, or posterior third of the corneal stroma. When the Descemet membrane was bared then a Descemetocoele was declared. A corneal perforation was present if fluorescein staining showed leakage of aqueous occurring through the Descemetocoele or the anterior chamber was partially or completely collapsed.

Animals were randomly subdivided into three groups of 16 eyes each: (1) PBS control, (2) 800 μ M (D)-RTR tetramer in PBS alternating each hour with 1.5 mM (L)-RTR tetramer in PBS, and (3) 5F (12 μ M) in PBS. Each animal received one drop of the appropriate medication 2 hours after the alkali injury. Drops were repeated once every hour for 14 hours a day for 33 days and then treatment was discontinued until the end of the experiment on day 42. The frequency of ulceration between groups was evaluated by calculating the mean \pm SEM and testing for significance using the χ^2 test. *P* < 0.05 was considered significant. The severity of ulceration between groups was evaluated

by the Mann-Whitney test. Results are expressed as mean \pm SEM, unless otherwise stated.

RESULTS

In Vitro Experiments

The complementary sequence, RTR, was designed to specifically interact hydrophatically with the PGP sequence and, therefore, should interact with both Ac-PGP and Me-PGP. The (D)-RTR tetrameric peptide was designed to also inhibit both PGP chemoattractants, but have a greater stability in vivo by resisting proteolytic degradation. The following preliminary studies were performed in vitro to confirm the activity of each inhibitor before its use in the alkali-injured rabbit eye.

(L)- or (D)-RTR Tetramer Inhibition of Neutrophil Polarization

Neutrophil polarization in the presence of 800 μ M Ac-PGP was increased 50% above the control level. (L)- and (D)-RTR tetramer produced comparable inhibitions of this Ac-PGP induced neutrophil polarization (Table 1). Neutrophil polarization in the presence of 1 mM Me-PGP was increased 50% above the control level. Both RTR tetramers produced similar inhibitions of this Me-PGP induced neutrophil polarization (Table 2). Inhibition of Ac-PGP was two orders of magnitude greater than inhibition of Me-PGP for both tetrameric peptides.

Effect of 5F on Ac-PGP-Activated Neutrophil Polarization

Synthetic 5F did not significantly affect Ac-PGP activation of neutrophil polarization (800 μ M Ac-PGP: 41% \pm 2.7%, *n* = 5; 800 μ M Ac-PGP + 10 μ M 5F: 49% \pm 4.9%, *n* = 5).

TABLE 2. Inhibition of Me-PGP-Induced Neutrophil Polarization

Complementary Peptide	5 μ M	70 μ M	500 μ M	ID ₅₀	P
(L)-RTR tetramer	—	60% \pm 29.7% (<i>n</i> = 2)	100% (<i>n</i> = 2)	57 μ M	<0.01
(D)-RTR tetramer	14% \pm 4.5% (<i>n</i> = 5)	45% \pm 4.9% (<i>n</i> = 2)	100% (<i>n</i> = 5)	110 μ M	<0.001

Untreated neutrophils (negative control) produced a polarization response of 7% \pm 1.5% (*n* = 3). Neutrophils activated with 1 mM Me-PGP (positive control) produced a polarization response of 58% \pm 5.5% (*n* = 11). This concentration of the PGP chemoattractant was selected from the linear portion of the dose-response curve, yielding approximately 50% polarization after subtraction of the negative control values. Percent inhibition (mean \pm SEM) of Me-PGP induced neutrophil polarization for each concentration of both RTR tetramers was calculated after subtraction of the negative control from all values. Each ID₅₀ was interpolated from the RTR tetramer dose-response curve as the concentration required to produce 50% inhibition of the positive control. Student's *t*-test was used to analyze the statistical difference between the polarization response produced by the highest concentrations of each RTR tetramer (70 μ M plus 500 μ M (L)-RTR tetramer or 500 μ M (D)-RTR tetramer) in the presence of 1 mM Me-PGP and the polarization response produced by the positive control (1 mM Me-PGP-activated neutrophils). ID₅₀, 50% inhibitory dose.

Effect of 5F on Neutrophil Respiratory Burst

Neutrophils exposed to opsonized zymosan produced a significantly greater respiratory burst than control neutrophils. The synthetic 5F peptide significantly inhibited this opsonized zymosan induced stimulation (Fig. 1A). Neutrophils exposed to the respiratory burst stimulant derived from alkali-degraded corneas produced a significantly greater respiratory burst than

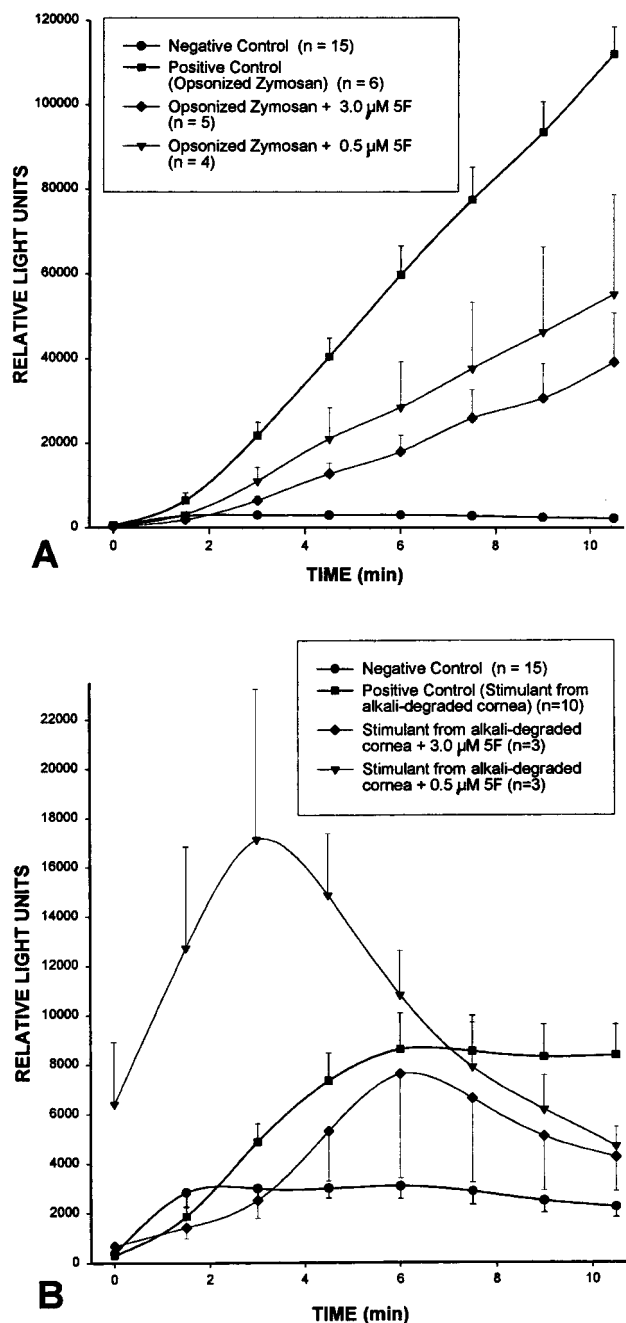


FIGURE 1. The effect of 5F on the respiratory burst of neutrophils. Negative controls consisted of neutrophils alone. 5F alone had no effect on resting neutrophils (negative control). Positive control samples consisted of neutrophils exposed to opsonized zymosan (600 ng/ml) or the respiratory burst stimulant derived from alkali-degraded corneas (1:50 dilution). (A) Synthetic 5F significantly inhibited the opsonized-zymosan-induced respiratory burst of neutrophils. Each data point represents the mean \pm SEM. (B) Synthetic 5F failed to inhibit neutrophil activation by the respiratory burst stimulant derived from alkali-degraded corneas. Each data point represents the mean \pm SEM. Significance was calculated with Student's *t*-test.

control neutrophils. Synthetic 5F failed to inhibit this respiratory burst (Fig. 1B). In fact, 5F significantly enhanced the neutrophil respiratory burst triggered by this stimulant in the first 5 minutes.

In Vivo Experiment

We had planned to terminate the experiment on day 33, therefore we prepared the highest possible concentrations of the RTR tetramers to cover that time interval. It was only apparent toward the very end of the experiment that it was unwise to terminate the experiment on day 33, because significant clinical changes were continuing in the rabbit model. The unavailability of additional RTR tetramer at this juncture, however, required that the application of RTR tetramer drops be discontinued at day 33. The clinical results at day 33 showed a significant reduction in the frequency of corneal ulceration in the RTR tetramer group compared with the PBS group (Table 3). From the beginning of the experiment until day 33, 9 ulcers developed in the PBS group, 11 ulcers in the 5F group (not significant), and 4 ulcers in the RTR tetramer group ($P = 0.0360$). During the remaining time of the experiment (days 33–42), when no further topical drops were applied to any animal group, several new ulcers appeared. Despite cessation of all drops at day 33 the favorable effect of the RTR inhibitor persisted to the end of the experiment. Considering all ulcers throughout the experiment, statistically fewer ulcers occurred in the RTR tetramer group than in the PBS control group (43.8% vs. 87.5%, $P = 0.0046$).

The severity of corneal ulcers was statistically different at day 42 ($P < 0.05$) in the RTR-tetramer group when compared with the control group, but the severity of ulcers in the RTR-tetramer group was no different from control ulcers at day 33 (Fig. 2). Although corneal ulceration was significantly more severe in the 5F group than the control PBS group throughout the majority of the experiment, this difference began to disappear after day 30 and the two groups were almost identical by the end of the experiment.

There were no significant differences among the three groups in the size of epithelial defects or corneal neovascularization.

DISCUSSION

In previous ocular alkali injury studies, we demonstrated that ascorbate enhances the repair mechanism by the production of new collagen in the cornea.^{22,23} Another very productive approach to damage control has been interference with neutrophil function. Corneal ulcers developing after alkali injury are consistently associated with a heavy infiltration of neutrophils. Exclusion of PMNs has been shown to protect the injured corneal stroma from the degradative enzymes and oxygen free radicals released from these inflammatory cells. Directing treatment at the PMN by the chelating agent citrate inhibits all neutrophil activities *in vitro*^{16,24–26} and significantly reduces corneal ulceration *in vivo*.^{27–31} Combining ascorbate and citrate treatments effected a 4% incidence of transient, shallow corneal ulcers; the best response of any treatment to date.³² A synthetic metalloproteinase inhibitor reduced corneal ulceration in the alkali-injured eye to a degree similar to citrate or ascorbate alone, but inferior to the combination of ascorbate and citrate.^{33–35}

The current work concentrates on the earliest step in this complex inflammatory process. Inactivation of the PGP neutrophilic chemoattractants released in the first hours after alkali injury. The concept of applying an antisense inhibitor to the alkali-injured eye designed specifically to inactivate a neutrophil chemoattractant is an initial effort to develop a completely novel approach to this disastrous injury. This experiment dem-

TABLE 3. Clinical Results of the Treatment of Alkali-Injured Rabbit Eyes

	RTR Tetramer	PBS	5F	P
Number of eyes	16	16	16	
Total ulcers from Day 1 to Day 33 (%)	4 (25)	9 (56.3)	11 (68.8)	$P = 0.0360$
Ulcers healed (<i>n</i>)	1	2	3	
New ulcers from Day 33 to Day 42	3	5	0	
Total ulcers at Day 42 (%)	6 (37.5)	12 (75)	8 (50.0)	$P = 0.0163$
Total ulcers during study period (%)	7 (43.8)	14 (87.5)	11 (68.8)	$P = 0.0046$
Ulcers depth during study period (<i>n</i>)				
Anterior	4	6	1	
Middle	0	3	3	
Posterior	2	4	2	
Descemetocoele	1	1	5	

Probabilities are by $[\chi]^2$ test and compare results in RTR-treated eyes with those in control (PBS-treated) eyes.

onstrates that (L)- and (D)-RTR tetramers, used alternately in the same eye, significantly reduced the incidence of corneal ulcers occurring after alkali injury. The reduction in corneal ulceration is not as great as that noted after citrate, ascorbate or metalloproteinase inhibitors, but represents the beginning of the investigative process to optimize the RTR tetramer.

Ac-PGP and Me-PGP are the primary neutrophil chemoattractants found in the stroma as a consequence of direct hydrolysis of corneal proteins immediately after an alkali injury.^{4-6,34} The favorable results obtained in this animal experiment are predicated on the inhibitory effect of the RTR tetramer on the synthetic PGP chemoattractants in vitro. It is likely that the complementary binding of RTR tetramer to Ac-PGP and Me-PGP partially neutralized these chemoattractants, thereby significantly reducing the neutrophilic response in the cornea. A direct histologic evaluation of the effect of RTR tetramer on PMN infiltration, in vivo, is planned.

The delivery of higher concentrations of the RTR tetramer into the corneal stroma would probably provide even greater benefit to the alkali-injured eye. The drop solutions for the current in vivo experiment consisted of 1.5 mM (L)-RTR tetramer and 750 μ M (D)-RTR tetramer, alternating every hour. These concentrations of RTR tetramer were dictated by the limited quantities of each synthetic RTR tetramer available.

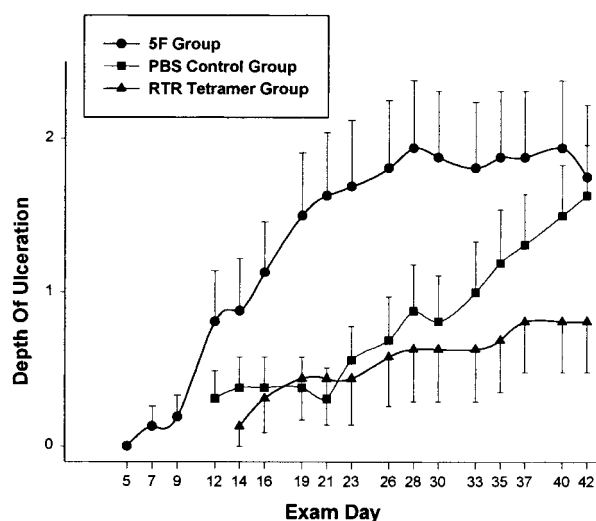


FIGURE 2. Progression of corneal ulcer severity over the course of the experiment. Each increasing depth of ulceration was assigned an increasing whole number from 0 to 5 (no ulcer, 0; anterior, 1; middle, 2; posterior, 3; Descemetocoele, 4; and perforation, 5). Each point represents the mean grade of ulcer severity for all 16 eyes in that group on that day, after alkali injury. Statistical significance was determined with the Mann-Whitney test.

Analysis of a prior experiment on the penetration of citrate into the de-epithelialized rabbit cornea³⁶ suggests that the RTR peptide solutions used in the present study may have been suboptimal. The stromal concentrations of the (L)- and (D)-RTR tetramers, resulting from topical drops, were probably lower than the previously reported in vitro concentration of (L)-RTR tetramer (30 μ M) required to produce 90% inhibition of neutrophils activated by the partially purified PGP chemoattractant fraction obtained from alkali-degraded corneas.¹¹ The optimal RTR tetramer concentration for in vivo activity is unknown.

Comparison of in vitro data on (L)- and (D)-RTR tetramers from this present study with (L)-RTR tetramer data from a previously published study¹¹ shows similar inhibitions of neutrophils exposed to synthetic PGP chemoattractants. In the current and previous study, the (L)-RTR tetramer concentration required to produce a 90% inhibition of neutrophils in vitro was 0.5 μ M and 1 μ M for synthetic Ac-PGP peptide, but thirty times higher when activated by the partially purified PGP chemoattractant fraction obtained from alkali-degraded corneas (30 μ M).¹¹ We believe the reason for this disparity is that, in addition to Ac-PGP and Me-PGP, the purified alkali-degraded corneal sample contains numerous inactive peptides having a 100 to 1000 MWt. These inactive peptides may interfere, non-specifically, with the RTR tetramer inhibition. Given that only a minute fraction of the peptides are chemoattractants in the alkali-injured cornea it would be expected that a higher concentration of the (L)-RTR tetramer (>30 μ M) would be required to produce more than 90% inhibition in vivo. Dose-response curves are planned for the RTR tetramer in vivo.

The statistically significant reduction in the frequency of ulceration in the RTR tetramer group compared with the PBS control group persisted from day 33 ($P = 0.0360$) to the end of the experiment ($P = 0.0163$), despite cessation of RTR-tetramer therapy at 33 days. Early blocking of the PGP chemoattractants may have reduced the major stimulus to further neutrophilic invasion, providing protection to the cornea. These considerations indicate that early treatment of the alkali-injured eye by RTR tetramer may be mandatory to inhibit corneal ulcer formation.

Three new ulcers formed in the RTR group after 33 days, whereas five formed in the control group. This finding combined with the earlier discussion of suboptimal concentrations of RTR tetramer suggests that the alkali-derived PGP chemoattractants, generated immediately by alkaline hydrolysis of corneal proteins, may have been incompletely neutralized by the RTR tetramers. The early PMN response begins before 12 hours and builds to a peak at approximately 2 days before further recruitment of neutrophils is noted.³⁷ In point of fact, we do not know when the Ac-PGP and Me-PGP peptides peak and when they no longer play a part in the chemoattraction of neutrophils. These molecules may remain in the corneal stroma much longer than imagined. Nevertheless, it is logical

to believe that it is important to begin treatment within 24 hours and even within several hours. This is completely feasible clinically, but the logistics for delivering the medication must be in place beforehand.

Both the (L) and (D) forms of the RTR tetramer were used in this experiment to guard against the potential enzymatic degradation of the (L)-RTR sequence in the alkali-injured cornea. The paucity of any cells in the cornea during the first couple of days after the injury augurs low enzymatic activity. In subsequent days the influx of cells may produce enzymes capable of degrading the (L)-RTR sequence. The stability of (D)-amino acid peptides in vivo and the similar biological activities of (L)- and (D)-amino acid peptides provide the rationale for administering both tetramers.³⁸⁻⁴¹ Future experiments are needed to distinguish between the effectiveness of the (L)- and (D)-RTR tetramers.

Apo A-1 has been reported to inhibit receptor-activated respiratory burst and degranulation of PMNs in the presence of some inflammatory mediators, but has no effect on PMN chemoattractants.¹² In the current in vitro experiments, the apo A-1 mimetic peptide, 5F (7300 MWt), had no effect on the Ac-PGP chemoattractant but produced a significant inhibition of the neutrophil respiratory burst in the presence of opsonized zymosan. However, these same experiments demonstrate the inability of 5F to inhibit the respiratory burst of neutrophils activated with a metabolic stimulant derived from alkali-degraded corneas.^{42,43} In fact, this respiratory burst was significantly increased during the first 5 minutes. The in vitro failure of 5F to inhibit both mediators derived from alkali-degraded corneas and, in particular, its initial enhancing effect on the respiratory burst stimulant may explain the occurrence of more severe ulcers in the 5F-treated animals and the failure of 5F to reduce the frequency of ulcer formation.

This article represents an important step in conceptualizing, producing, and testing a drug inhibitory to chemoattractant peptides found in alkali-injured eye. Such an approach is intended to begin an iterative process in the development of more potent peptides that are able to stem the invasion of neutrophils into the cornea. Although other compounds have been found to be more potent, in most cases, there is little room for further development to enhance drug activity. In contrast, sense-antisense technology offers an opportunity to manipulate the peptide compounds in a completely new class of inhibitors. The results of this article should therefore be interpreted in the context of the first phase in the development of an entirely new concept in the treatment of the alkali-injured eye.

Acknowledgments

The authors thank G. M. Anantharamaiah, MD, University of Alabama at Birmingham, for synthesis of the peptides Ac-PGP and 5F and the University of Alabama Birmingham for the use of the Glycoprotein Analysis Core Facility, where the amino acid analysis of Ac-PGP was performed. We also thank Boopathy Dhanapal, Lipal Biochemicals, for the synthesis of (L)-RTR tetramer.

References

- Brown SI, Weller CA. The pathogenesis and treatment of collagen-induced diseases of the cornea. *Trans Am Acad Ophthalmol Otolaryngol.* 1970;74:375-383.
- Kenyon KR, Berman M, Rose J, Gage J. Prevention of stromal ulceration in the alkali-burned rabbit cornea by glued-on contact lens: evidence for the role of polymorphonuclear leukocytes in collagen degradation. *Invest Ophthalmol Vis Sci.* 1979;18:570-587.
- Foster CS, Zelt RP, Mai-Phan T, Kenyon KR. Immuno-suppression and selective inflammatory cell depletion: studies of a guinea pig model of corneal ulceration after ocular alkali burning. *Arch Ophthalmol.* 1982;100:1820-1824.
- Pfister RR, Haddox JL, Sommers CI, Lam K-W. Identification and synthesis of chemotactic tripeptides from alkali-degraded whole cornea: a study of *N*-acetyl-proline-glycine-proline and *N*-methyl-proline-glycine-proline. *Invest Ophthalmol Vis Sci.* 1995;36:1306-1316.
- Pfister RR, Haddox JL, Sommers CI. Alkali-treatment of cornea generates a low molecular weight chemoattractant for polymorphonuclear leukocytes. *Invest Ophthalmol Vis Sci.* 1993;34:2297-2304.
- Pfister RR, Haddox JL, Sommers CI. Injection of chemoattractants into normal cornea: a model of inflammation after alkali-injury. *Invest Ophthalmol Vis Sci.* 1998;39:1744-1750.
- Blalock JE. Genetic origins of protein shape and interaction rules. *Nat Med.* 1995;1:876-878.
- Blalock JE, Smith EM. Hydropathic anti-complementarity of amino acids based on the genetic code. *Biochem Biophys Res Commun.* 1984;121:203-207.
- Blalock JE. Complementarity of peptides specified by "sense" and "antisense" strands of DNA. *Trends Biotechnol.* 1990;8:140-144.
- Clarke BL, Blalock JE. Characteristics of peptides specified by antisense nucleic acids. In: vandrol Krol AV, Mol JNM, eds. *Antisense Nucleic Acids and Proteins: Fundamentals and Applications.* New York: Dekker; 1991:169-186.
- Pfister RR, Haddox JL, Blalock JE, Sommers CI, Coplan L, Villain M. Synthetic complementary peptides inhibit a neutrophil chemoattractant found in the alkali-injured cornea. *Cornea.* 2000;19:384-389.
- Blackburn WD Jr, Dohlman JG, Venkatachalapathi YV, et al. Apolipoprotein A-1 decreases neutrophil degranulation and superoxide production. *J Lipid Res.* 1991;32:1911-1918.
- Kaiser E, Colescott RL, Bossinger CD, Cook PI. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal Biochem.* 1970;34:595-598.
- Garber DW, Datta G, Chaddha M, et al. A new synthetic class A amphipathic peptide analog protects mice from diet-induced atherosclerosis. *J Lipid Res.* 2001;42:545-552.
- Ferrante A, Thong YH. A rapid one-step procedure for purification of mononuclear and polymorphonuclear leukocytes from human blood using a modification of the Hypaque-Ficoll technique. *J Immunol Methods.* 1978;24:389-393.
- Pfister RR, Haddox JL, Harkins LE, Dodson RW. The effects of citrate on fMLP induced polymorphonuclear leukocyte stimulation and locomotion. *Cornea.* 1984/1985;3:183-188.
- Wacker WEC, Ulmer DD, Vallee BL. Metalloenzymes and myocardial infarction. II: Malic and lactic dehydrogenase activities and zinc concentrations in serum. *N Engl J Med.* 1956;255:449-456.
- Decker LA. Lactate dehydrogenase. *Worthington Enzyme Manual.* Freehold, NJ: Worthington Biochemical; 1977:19-22.
- Haston WS, Shields JM. Neutrophil leukocyte chemotaxis: a simplified assay for measuring polarizing responses to chemotactic factors. *J Immunol Methods.* 1985;81:229-237.
- Pfister RR, Haddox JL, Sommers CI. Effect of synthetic metalloproteinase inhibitor or citrate on neutrophil chemotaxis and the respiratory burst. *Invest Ophthalmol Vis Sci.* 1997;38:1340-1349.
- Fraunfelder FT. Extraocular fluid dynamics; how best to apply topical ocular medication. *Trans Am Ophthalmol Soc.* 1976;74:457-487.
- Levinson RA, Paterson CA, Pfister RR. Ascorbic acid prevents corneal ulceration and perforation following experimental alkali burns. *Invest Ophthalmol Vis Sci.* 1976;15:986-993.
- Pfister RR, Paterson CA. Additional clinical and morphological observations on the favorable effect of ascorbate in experimental ocular alkali burns. *Invest Ophthalmol Vis Sci.* 1977;16:478-487.
- Pfister RR, Haddox JL, Dodson RW, DeShazo W. Polymorphonuclear leukocytic inhibition by citrate, other metal chelators and trifluoperazine: evidence to support calcium binding protein involvement. *Invest Ophthalmol Vis Sci.* 1984;25:955-970.
- Pfister RR, Haddox JL, Dodson RW, German VP. The effects of citrate and other compounds on PMN incubated in vitro: further studies on the site and mechanism of action of citrate. *Cornea.* 1984/1985;3:240-249.
- Pfister RR, Haddox JL, Snyder TL. The effects of citrate on the adherence of neutrophils to nylon fibers in vitro. *Invest Ophthalmol Vis Sci.* 1988;29:869-875.

27. Pfister RR, Nicolario ML, Paterson CA. Sodium citrate reduces the incidence of corneal ulcerations and perforations in extreme alkali-burned eyes: acetylcysteine and ascorbate have no favorable effect. *Invest Ophthalmol Vis Sci.* 1981;21:486-490.
28. Pfister RR, Haddox JL, Paterson CA. The efficacy of sodium citrate in the treatment of severe alkali burns of the eye is influenced by the route of administration. *Cornea.* 1982;1:205-211.
29. Pfister RR, Haddox JL, Snyder TL. Topical citrate inhibits the adherence of neutrophils to postcapillary venules. *Cornea.* 1990;9:238-245.
30. Williams RN, Paterson CA. Accumulation of polymorphonuclear leukocytes in ocular tissues in response to bacterial endotoxin: the inhibitory effect of sodium citrate. *Ocul Inflamm Ther.* 1983;1:103-109.
31. Haddox JL, Pfister RR, Yuille-Barr D. The efficacy of topical citrate after alkali injury is dependent on the period of time it is administered. *Invest Ophthalmol Vis Sci.* 1989;30:1062-1068.
32. Pfister RR, Haddox JL, Yuille-Barr D. The combined effect of citrate/ascorbate treatment in alkali-injured rabbit eyes. *Cornea.* 1991;10:100-104.
33. Burns FR, Gray RD, Paterson CA. Inhibition of alkali-induced corneal ulceration and perforation by a thiol peptide. *Invest Ophthalmol Vis Sci.* 1990;31:107-114.
34. Lee Y-C, Jackson PL, Jablonsky MJ, et al. NMR conformational analysis of *cis* and *trans* proline isomers in the neutrophil chemoattractant, N-acetyl-proline-glycine-proline. *Biopolymers.* 2001;58:548-561.
35. Paterson CA, Wells JG, Koklitis PA, Higgs GA, Docherty JP. Recombinant tissue inhibitor of metalloproteinases type 1 suppresses alkali-burn-induced corneal ulceration in rabbits. *Invest Ophthalmol Vis Sci.* 1994;35:677-684.
36. Pfister RR, Haddox JL, Paterson CA. The efficacy of sodium citrate in the treatment of severe alkali burns of the eye is influenced by the route of administration. *Cornea.* 1982;1:205-211.
37. Paterson CA, Williams RN, Parker AV. Characteristics of polymorphonuclear leukocyte infiltration into the alkali burned eye and the influence of sodium citrate. *Exp Eye Res.* 1984;39:701-708.
38. Van Regenmortel MH, Muller S. D-peptides as immunogens and diagnostic reagents. *Curr Opin Biotechnol.* 1998;9:377-382.
39. Fassina G, Cassani G, Corti A. Binding of human tumor necrosis factor alpha to multimeric complementary peptides. *Arch Biochem Biophys.* 1992;296:137-143.
40. Fassina G, Consonni R, Zetta L, Cassani G. Design of hydrophatically complementary peptides for big endothelin affinity purification. *Int J Peptide Protein Res.* 1992;39:540-548.
41. Fassina G, Roller PP, Olson AD, Thorgeirsson SS, Omichinski JG. Recognition properties of peptides hydrophatically complementary to residues 356-375 of the c-ras protein. *J Biol Chem.* 1989;264:11252-11257.
42. Pfister RR, Haddox JL, Lam KW. Alkali-degraded cells generate a respiratory burst stimulant for neutrophils. *Cornea.* 1993;12:155-160.
43. Pfister RR, Haddox JL, Yuille-Barr D, Berry S, Lam KW. Amino acid composition of a neutrophil respiratory burst stimulant; evidence for a protein, noncollagenous source. *Invest Ophthalmol Vis Sci.* 1991;32:2112-2118.

E R R A T U M

Erratum in: "Novel Cytochrome P4501B1 (*CYP1B1*) Gene Mutations in Japanese Patients with Primary Congenital Glaucoma" by Mashima et al. (*Invest Ophthalmol Vis Sci.* 2001;42:2211-2216).

On p. 2213, some numbers in Table 1 were transposed. The corrected table appears below, with the correct numbers in bold.

The online version of this article was corrected on October 3, 2001.

TABLE 1. Summary of Mutations in the *CYP1B1* Gene of Affected Individuals in 11 Japanese Families with Primary Congenital Glaucoma

Number of Families	Mutation DNA Change (Predicted Effect)	Mutation DNA Change (Predicted Effect)
3	4776insAT (frameshift)	G7927A (Val364Met)
1	A4380T (Asp192Val)*	
1	A4380T (Asp192Val)	G7927A (Val364Met)
1	G4793T, C4794T (Ala330Phe)	G7927A (Val364Met)
1	G7927A (Val364Met)	G8168A (Arg444Gln)
1	C4645A (Cys280stop)	G8168A (Arg444Gln)
1	3964delC (frameshift)	G8168A (Arg444Gln)
2	C3130T (unknown)†	G4763T (Val320Leu)
1	G4397A (Val198Ile)‡	Unidentified
1	A8333G (Glu499Gly)‡	Unidentified

* Homozygous.

† Probable mutation in the noncoding region of exon 1.

‡ Probable mutation found in one of two alleles.