Investigative Ophthalmology & Visual Science, Vol. 32, No. 7, June 1991

Amino Acid Composition of a Neutrophil Respiratory Burst Stimulant

Evidence for a Protein, Noncollagenous Source

Roswell R. Pfister,* Jeffrey L. Haddox,* Dena Yuille-Barr,* Susan Berry,† and Kwok-Wai Lam†

Activation of the neutrophil respiratory burst by the supernatant fraction from an alkali-treated collagen preparation (SAC) was enhanced by longer durations of exposure to alkali (1 N NaOH for 0.5–24 hr). The concentrate obtained from ultrafiltration (>30,000 molecular weight) of SAC (1 N NaOH for 24 hr) retained the stimulatory factor. Fractionation of this ultraconcentrate by high-performance liquid chromatography showed that the stimulatory activity resided in the void volume (highest molecular weight). The amino acid composition of this active fraction revealed that this proteinaceous stimulant was not derived from the collagen molecule. Treatment of the SAC with ultrapure bacterial collagenase increased its stimulatory capacity, confirming its noncollagenous nature. Alkali treatment of whole cornea also released a similar large molecular weight, noncollagenous protein that stimulated the respiratory burst of polymorphonuclear leukocytes. Enhanced stimulation after prolonged NaOH treatment of the collagen preparation or collagenase treatment of SAC suggests that the stimulant might reside between collagen fibrils and then be released as the matrix is degraded. Invest Ophthalmol Vis Sci 32:2112–2118, 1991

The development of corneal ulceration after alkali injury to the eye closely parallels the numbers and activity of polymorphonuclear leukocytes (PMNs or neutrophils) present in the stroma.[1-5] The sequence and nature of neutrophil activities are determined by inflammatory mediators which begin by enhancing their adherence to the vascular endothelium followed by chemotaxis into the injured tissue. This process culminates in a respiratory burst with cellular degranulation leading to the release of hydrolytic enzymes. We previously showed that alkali treatment of collagen preparations or corneal stroma stimulated neutrophil locomotion in the presence of albumin and induced respiratory burst activity when albumin was absent.[6-8] In high concentrations, the respiratory stimulant triggered neutrophil lysis, causing the release of their full complement of hydrolytic enzymes.

We suggested that these neutrophilic stimulants might play important roles in mediating the inflammatory process in alkali-injured corneas. In our in vitro model of alkali-treated collagen preparations (1 N NaOH for 30 min at 35°C), we determined that the stimulant to the neutrophil respiratory burst was a large macromolecule (>200,000 daltons).[7] In this study, we tried to determine the chemical composition of the stimulant and whether it was derived from the collagen molecule.

From the *Eye Research Laboratories, AMI/Brookwood Medical Center, Birmingham, Alabama, and the †Department of Ophthalmology, University of Texas Health Science Center at San Antonio, San Antonio, Texas.

Supported by National Eye Institute grant EY-04716, AMI/Brookwood Medical Center, San Antonio Area Foundation, and a development grant from the Research to Prevent Blindness, Inc.

Submitted for publication: March 14, 1990; accepted February 9, 1991.

Reprint requests: Roswell R. Pfister, MD, Suite 211, 2008 Brookwood Medical Center Drive, Birmingham, AL 35209.

Materials and Methods

Materials

Type I insoluble collagen (catalog C-4387, lot 115F-0318), Ficoll, bovine serum albumin (BSA), and ultrapure bacterial collagenase were purchased from Sigma (St. Louis, MO). Whole frozen bovine corneas were obtained from Pel-Freez Biologicals (Rogers, AR). Ultrapure bacterial collagenase also was obtained from Advance Biofactures (Lynbrook, NY). Hypaque was purchased from Winthrop Pharmaceuticals (New York, NY) and Hank's balanced salt solution (HBSS) was obtained from GIBCO Laboratories (Grand Island, NY).

Alkali Treatment of Collagen Preparation

Collagen preparations were treated with alkali for 0.5, 1, 18, or 24 hr at 37°C. A total of 6 ml of 1N NaOH was added to 1 g of collagen. Each sample was neutralized subsequently with 1 N HCl and then cen-

trifuged at 15,000 × g for 10 min. Microliter volumes of the supernatant fractions from alkali-treated collagen preparations (SAC) were added to the PMN incubation mixtures to test for stimulation of the neutrophil respiratory burst.

Isolation of the Stimulant From SAC

The above studies demonstrated that the greatest neutrophil stimulation was obtained when the collagen preparations were treated with alkali for 24 hr. All subsequent studies used this exposure time as the initial step in the purification process. The SAC from this sample was concentrated (threefold) by ultrafiltration. This ultraconcentrate was separated according to molecular size by high-performance liquid chromatography (HPLC). The fractions were tested for stimulation of the neutrophil respiratory burst. The SAC, ultraconcentrate, and active HPLC fraction were compared for respiratory burst activity and relative protein concentrations by the Lowry method[9] and analyzed for amino acid composition.

Alkali Treatment and Isolation of Stimulant From Whole Cornea

Whole corneas were treated with 1 N NaOH (1 g dry weight/12 ml of 1 N NaOH) for 24 hr at 37°C. The supernatant fraction from whole cornea was carried through the ultrafiltration procedures and rinsed with HBSS to a final concentration of 14-fold. The subsequent techniques using Lowery protein analysis, HPLC, and amino acid composition were the same as noted for the SAC analysis.

Ultrafiltration

The SAC (9.2 ml) was centrifuged at 2000 × g for 1 hr in a Centriprep ultrafiltration apparatus containing a 30,000 molecular weight cutoff membrane (Amicon, Danvers, MA). The filtrate (8.2 ml) showed no respiratory burst activity. Then 1 ml of the concentrate (>30,000 molecular weight) was washed in 14 ml of physiologic saline and recentrifuged. The final ultraconcentrate (threefold) was resuspended in 3 ml of physiologic saline.

HPLC

Samples were injected into columns supplied by Waters Associates (Milford, MA). The eluate was monitored by absorbance at 210 and 280 nm in a multiple wavelength ultraviolet light detector (Model 490; Waters).

Lowry Protein Analysis

Protein concentrations were analyzed by the method of Lowry et al[9] using BSA as the standard.

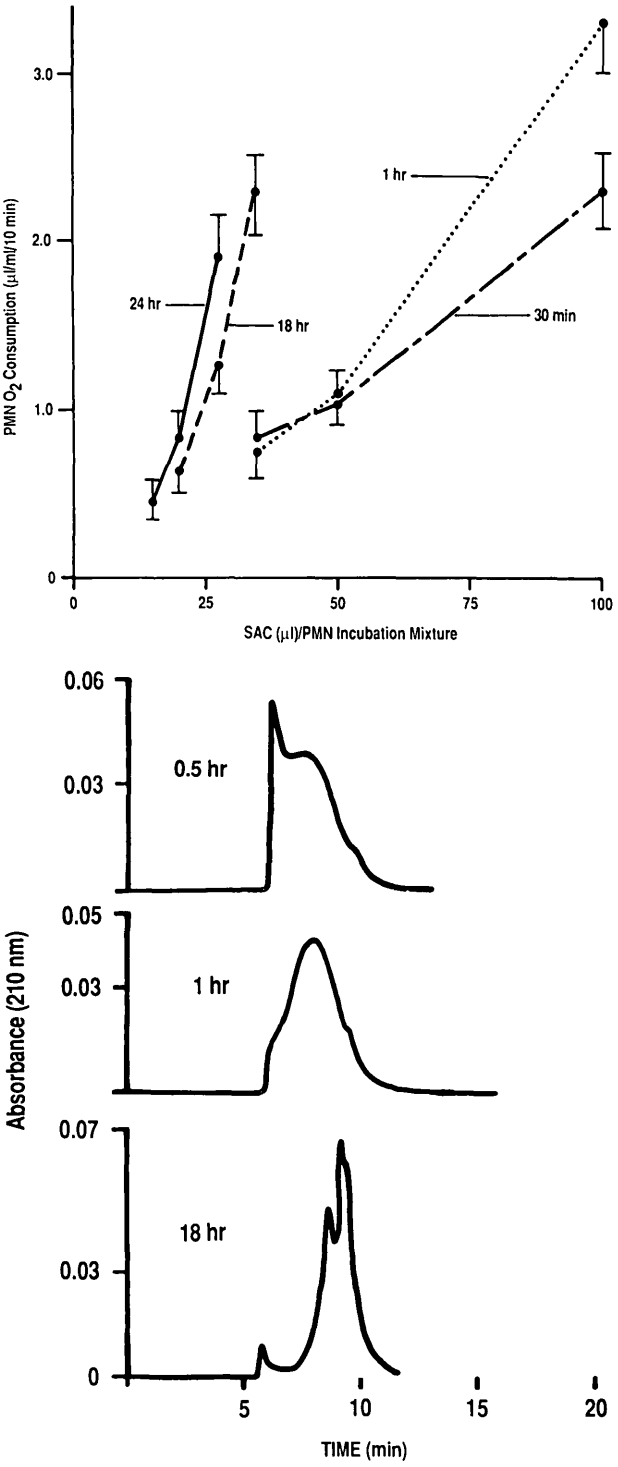
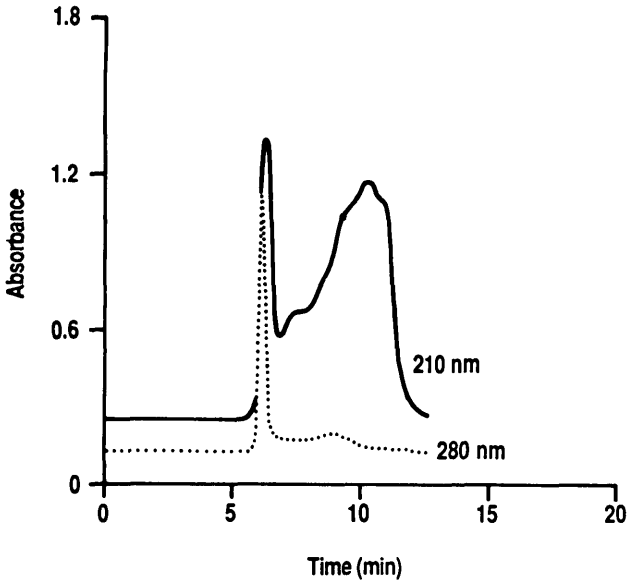
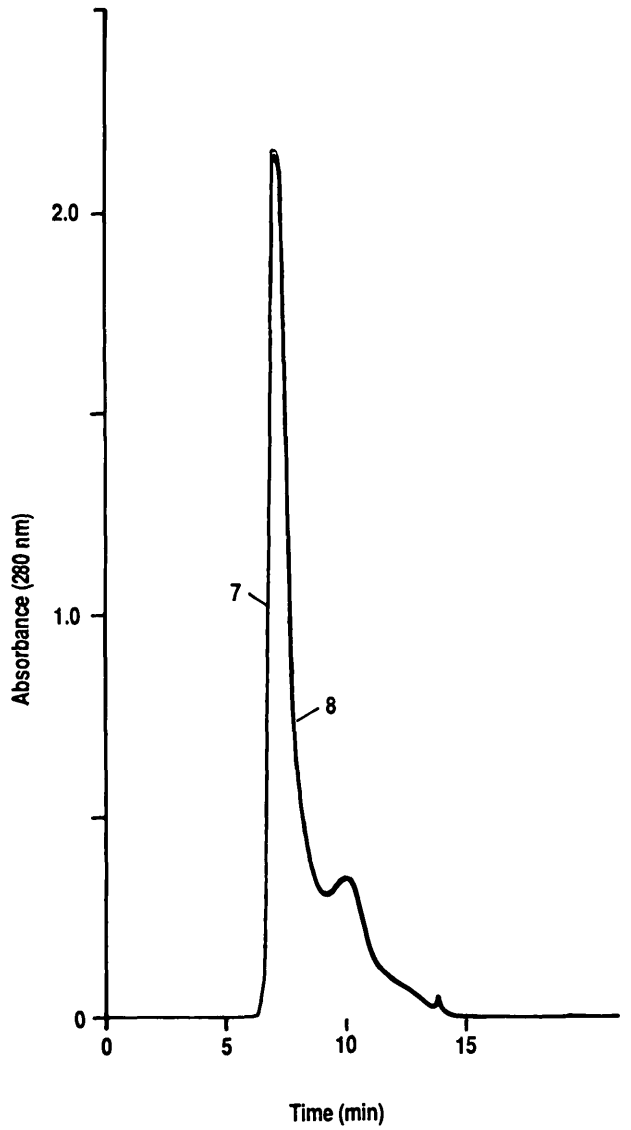


Fig. 1. Prolonged exposure of the collagen preparation to alkali increases the capacity of small amounts of SAC to stimulate the respiratory burst. (**A**) Increased stimulation of the neutrophil respiratory burst occurred after a 24-hr exposure of the collagen preparation to 1.0 N NaOH (w/v = 1.0 g/6 ml). A 100 µl of distilled water was added to the incubation mixtures that received 100 µl of SAC to maintain their osmolalities between 270 and 330 mOsm. Each data point consisted of 5 values. (**B**) The collagen preparation was treated with 1.0 N NaOH (10 mg/ml). These samples were diluted (1:100), and 1 µl was injected into a protein PAK-60 HPLC column and eluted by 0.1 M NaCl at a rate of 1 ml/min.





This assay underestimates the concentration of collagen and is satisfactory only when comparing relative protein concentrations.

Amino Acid Composition

Amino acid composition was analyzed by the picotag quantitation method using a reverse-phase picotag column from Waters Associates.

Treatment of SAC With Bacterial Collagenase

The SAC was treated with ultrapure bacterial collagenase (final concentration, 500 μg/ml) at 37°C for 5 hr, pH 7.5. The specific activity of collagenase (from *Clostridium histolyticum,* type VII) was 1920 units/mg protein. One unit will release peptides from native collagen equivalent in ninhydrin color to 1 μmol of L-leucine in 5 hr at pH 7.4 at 37°C in the presence of Ca^{2+}.

Microliter volumes of SAC, SAC after treatment with active or heat-inactivated bacterial collagenase, or enzyme alone were added to the PMN incubation mixtures to test for stimulation of the neutrophil respiratory burst. This experiment was repeated by incubating ultrapure bacterial collagenase (chromatographically pure form III) from Advance Biofactures with SAC and then testing for respiratory burst activity, yielding similar results.

Isolation of Neutrophils

Neutrophils were isolated from fresh human whole blood by centrifugation on Hypaque-Ficoll (density, 1.114, Winthrop Pharmaceuticals).[10,11] Isolated neutrophils were resuspended in HBSS to a purity of 91.3% ± 1.4% (mean ± standard error of the mean, n = 12) with a viability of 96–99%. The remaining percentage consisted of red blood cells and less than 5% platelets, lymphocytes, and eosinophils.

Neutrophil Respiratory Burst

The respiratory burst of neutrophils was measured with a Clark-type oxygen monitor (YSI model 53, Yellow Springs, OH) in an incubation chamber maintained at 37°C with a pH range of 7.2–7.6. Each incu-

Fig. 2. HPLC analysis and fractionation of the ultraconcentrate from SAC (1.0 N NaOH for 24 hr, w/v = 1.0 g/6 ml). (**A**) 50 μl of the ultraconcentrate was injected into an I-125 column. The sample size produced a cleaner separation of the high-molecular-weight peak from the residual low-molecular-weight components. Solid line, absorbance at 210 nm; dotted line, absorbance at 280 nm. (**B**) Ultraconcentrate (1.0 ml) was injected into a column (I-125) and eluted at 1.0 ml/min in 0.15 M saline. Each fraction was 1.0 ml. The location of the active fraction (7) was shown on the chromatogram.

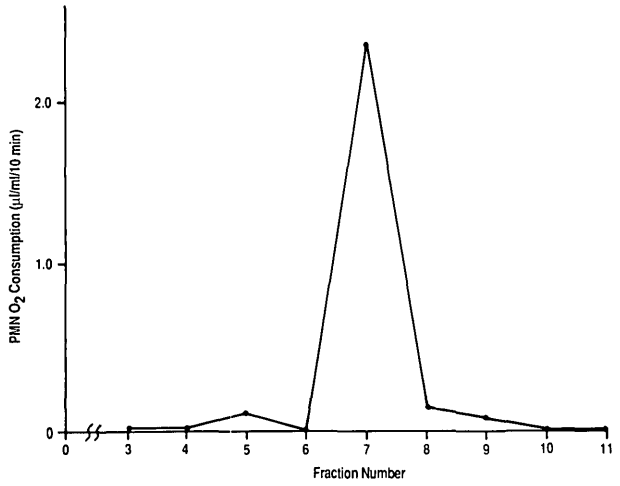


Fig. 3. After ultrafiltration, the ultraconcentrate from SAC (1.0 N NaOH for 24 hr, w/v = 1.0 g/6 ml) was separated by HPLC and the stimulant associated with the high-molecular-weight fraction (7) by testing the capacity of each fraction to stimulate the PMN respiratory burst. Each incubation mixture (1.0 ml) contained 75 µl of separate fractions from HPLC at isotonicity.

bation mixture contained 4.5×10^6 cells/ml HBSS and had final osmolalities ranging from 270–330 mOsm (unless otherwise noted). The final concentration of Ca^{2+} and Mg^{2+} in the incubation mixtures were 425–500 µM and 510–600 µM, respectively. Differing sample volumes were added to the cell suspensions to make a total incubation chamber volume of 1 ml. Oxygen consumption was measured for 10 min.

Statistics

The mean and standard error of the mean were used for data points throughout this study.

Results

Alkali Treatment of Collagen Preparations

Figure 1A shows that, as the period of exposure of collagen preparations to 1.0 N NaOH was increased from 30 min to 24 hr, much smaller volumes of SAC produced a similar stimulation of neutrophils. Results from HPLC of the collagen preparations treated with alkali for 30 min showed that an initial sharp peak (high molecular weight peptide) was followed by a broad peak. The high molecular weight material present in the first peak decreased as the incubation time increased. This indicated that most of the collagen present in the first peak was reduced to a heterogeneous collection of low molecular weight peptides (Fig. 1B). Significantly, a small residual amount of the high molecular weight species remained even after an 18-hr exposure.

Table 2. Amino acid composition of the purification steps of SAC

	Supernatant fraction (SAC) (%)	*Ultraconcentrate (3×) (%)*	**Active HPLC fraction (#7) (%)*
Aspartic acid	4.6	8.0	7.3
Glutamic acid	7.6	10.1	8.8
Hydroxyproline	7.2	4.3	0.4
Serine	2.9	3.6	5.9
Glycine	33.2	22.6	8.4
Histidine	0.4	1.2	2.5
Arginine	4.5	4.9	6.4
Alanine	12.5	9.5	7.8
Proline	16.1	13.0	9.6
Tyrosine	0.4	1.8	3.0
Methionine	0.4	1.1	1.5
Valine	2.2	4.4	8.8
Isoleucine	1.1	2.5	5.0
Threonine	0.5	1.2	2.3
Leucine	2.5	6.5	12.7
Phenylalanine	1.8	2.7	5.5
Lysine	2.2	2.7	4.3

* One milliliter of concentrate was injected into the HPLC column to produce this fraction.

Isolation of the Stimulant From SAC

Ultrafiltration of SAC (1 N NaOH for 24 hr) removed the 0.5 M NaCl and most of the small peptides from the concentrate (compare small molecular weight peaks, Figs. 1B [18 hr], 2A). The remaining small peptides were derived from collagen as evidenced by the peak at 210 nm but not at 280 nm (Fig. 2A). The appearance of the high molecular weight absorbance peak at both wavelengths suggests the stimulant was a noncollagenous protein(s). The ultra-

Table 1. Purification of the respiratory burst stimulant

	**Supernatant fraction (SAC)*	*Ultraconcentrate (3×)*	†*Active HPLC fraction (#7)*
Sample volume (ml)	9.2	3.0	1.0
Protein concentration (mg/ml)	44.1	4.5	0.9
‡PMN respiratory burst (μl O_2 consumed/ml/10 min)	2.6	1.3	0.9

* SAC (1 N NaOH for 24 hr).
† [illegible] ml of ultraconcentrate (×3) was injected into the HPLC column.
‡ 50 µl of stimulant sample was added to the PMN incubation mixture.

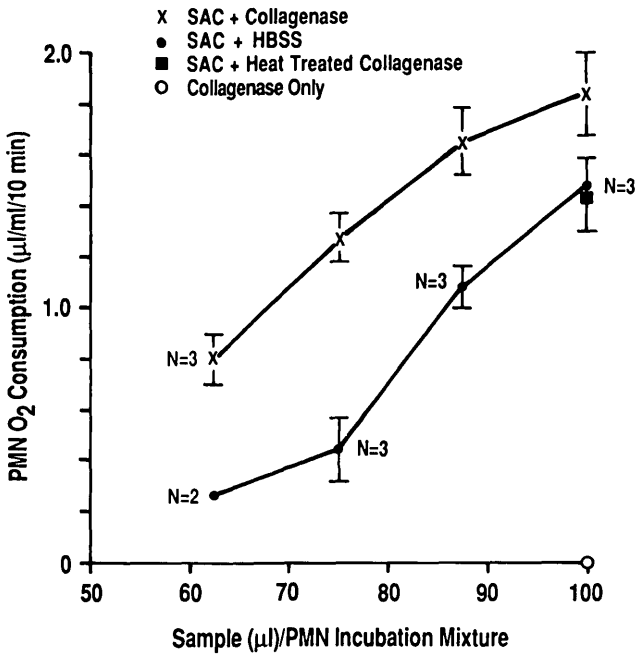


Fig. 4. Bacterial collagenase treatment of SAC (1 N NaOH for 24 hr, w/v = 1.0 g/6 ml) increased its capacity to induce stimulant activity. SAC was mixed (1:1) with the enzyme in HBSS (×), heat inactivated (100°C for 1 hr) enzyme in HBSS (solid square), or HBSS alone (solid circle). The enzyme was added alone to the PMN suspension as a negative control (open circle). Each data point consisted of four values unless otherwise noted.

filtrate did not activate the PMN respiratory burst. The ultraconcentrate retained the high molecular weight peak and contained the stimulant. A loss of respiratory burst activity occurred when the concentrated stimulant was resuspended in physiologic saline. The ultraconcentrate then was separated by HPLC fractionation (Fig. 2B). The stimulant was obtained in fraction 7 (Fig. 3) at the void volume (>125,000 molecular weight for native proteins and 30,000 for denatured proteins). The greater purity of fraction 7 over SAC was demonstrated by a large reduction in protein concentration and a much smaller decrease in the PMN respiratory burst (Table 1). The amino acid composition of samples from each purification step showed a stepwise reduction in the percentage of glycine and hydroxyproline (Table 2).

Treatment of SAC With Bacterial Collagenase

Treatment of the SAC with ultrapure bacterial collagenase increased its capacity to activate the PMN respiratory burst (Fig. 4).

Isolation of Stimulant From Whole Cornea

Whole corneas were virtually liquified after 24 hr exposure to 1 N NaOH. This suspension was neutralized with acid and centrifuged to remove visible debris. The supernatant fraction was ultrafiltered to concentrate large molecules and remove small molecules and hypertonic salt. Rinsing with HBSS during ultrafiltration produced an isotonic concentrate with less than one half of the original activity but more than 98% of the remaining activity. The concentrate retained large peptides, and most small peptides were removed (Fig. 5). After the concentrate was separated by HPLC, activity was recovered in fractions 7–10, peaking in 9 (Fig. 6). Purification of the stimulant was shown by a large reduction in protein concentration but no reduction in respiratory burst activity (Table

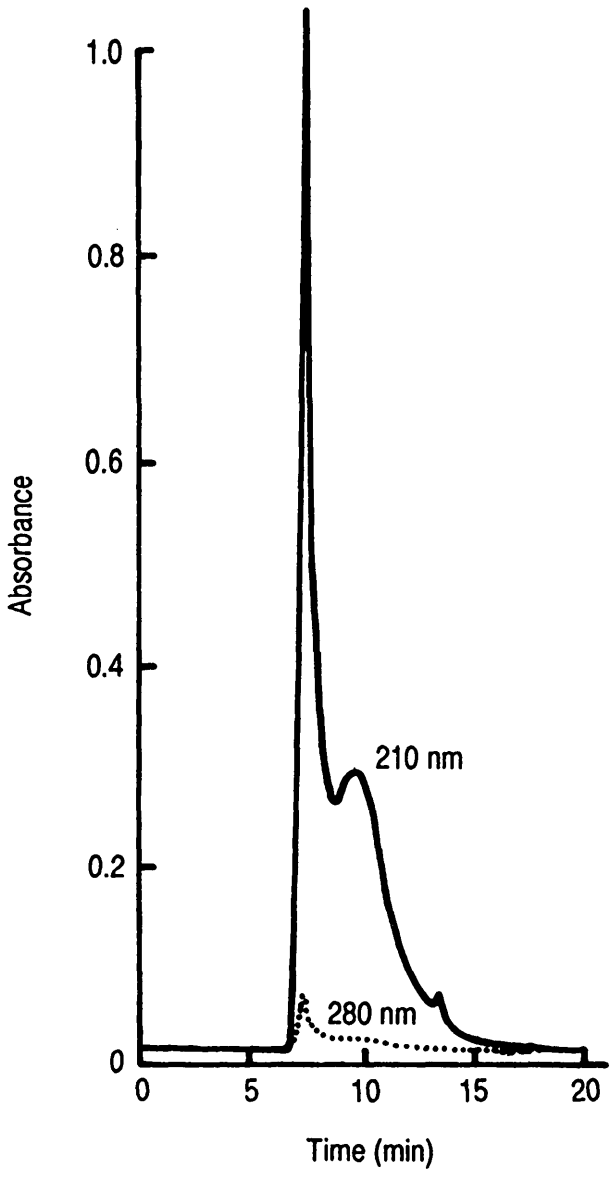


Fig. 5. HPLC analysis and fractionation of the ultraconcentrate from whole corneas. One microliter of the ultraconcentrate was injected into an I-125 column. The sample size produced a cleaner separation of the high-molecular-weight peak from the residual low-molecular-weight components. Solid line, absorbance at 210 nm; dotted line, absorbance at 280 nm.

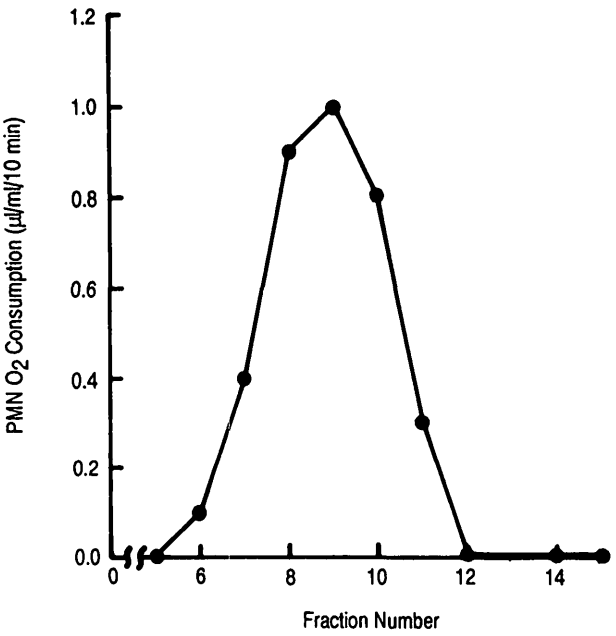


Fig. 6. Respiratory burst activity of PMNs responded to fractions from HPLC separation of the whole cornea ultraconcentrate. One milliliter was injected into the HPLC column (I-125) and eluted at 1.0 ml/min in 0.15 M saline. Each fraction was 1.0 ml, so that each fraction equaled the corresponding minute. Each fraction (75 µl) was tested for respiratory burst activity by adding to the 1.0 ml PMN incubation mixture. The presence of the respiratory burst stimulant was established in fractions 7–10, reaching its peak in 9. This broad peak was probably the result of high amounts of noncollagenous proteins, other than the stimulant, that remained in the ultraconcentrate.

3). Table 4 illustrates that there was a reduction in glycine and hydroxyproline as the stimulant became purer.

Discussion

Inflammatory mediators control the influx and metabolic state of neutrophils in damaged tissues. In a previous report we identified a neutrophil stimulant in corneal stroma and noncorneal type 1 collagen preparations that activated the respiratory burst in the absence of albumin and, in high concentrations, lysed the cells to release their granular enzymes.[6] A follow-up study determined that the stimulant was a macromolecule (>200,000 molecular weight).[7] We suggested that this stimulant might function as an inflammatory mediator in the alkali-injured eye.

Purification of the stimulant in this study was facilitated by prolonged treatment of the collagen preparation or whole cornea with alkali. Treatment for 24 hr increased the stimulatory capacity of SAC and degraded most of the collagen into much smaller, nonstimulatory polypeptides. The residual high molecular weight compound was more resistant to alkali treatment than collagen. Elimination of the lower molecular weight polypeptides by ultrafiltration increased the purity of the stimulant. Replacement of 0.5 M NaCl with physiologic saline during the ultrafiltration process resulted in a decreased respiratory burst, confirming previous findings that hypertonicity enhances the stimulatory effects of this agent.[6]

Table 4. Amino acid composition of the purification steps of whole cornea

	Supernatant fraction from whole cornea (%)	*Ultraconcentrate (14×) (%)*	**Active HPLC fraction (#8) (%)*
Aspartic acid	3.2	5.6	3.9
Glutamic acid	6.3	8.6	7.4
Hydroxyproline	3.9	0.6	0.4
Serine	3.9	4.9	5.4
Glycine	26.6	8.5	8.4
Histidine	0.6	1.8	0.9
Arginine	4.6	4.4	4.7
Alanine	10.2	6.9	7.0
Proline	14.4	8.0	8.8
Tyrosine	1.5	4.1	4.4
Methionine	1.3	2.6	2.4
Valine	4.2	7.8	8.2
Isoleucine	3.0	6.9	7.3
Threonine	1.6	2.5	2.9
Leucine	5.9	13.8	14.5
Phenylalanine	4.3	6.2	6.9
Lysine	4.7	7.1	6.7

* One ml of concentrate was injected into HPLC column.

Table 3. Purification of the respiratory burst stimulant from whole cornea

	**Supernatant fraction from whole cornea*	*Ultraconcentrate (14×)*	†*Active HPLC fraction (#8)*
Sample volume (ml)	42.0	3.0	1.0
Protein concentration (mg/ml)	97.3	114.3	13.7
‡PMN respiratory burst (μl O_2 consumed/ml/10 min)	0.8	2.7	0.9

* Whole cornea (1 N NaOH for 24 hr).
† One ml of ultraconcentrate (14×) was injected into the HPLC column.
‡ 60 µl of stimulant sample was added to the PMN incubation mixture.

The stimulant obtained from alkali treatment of whole cornea was greatest in fraction 9, although fractions 7–10 showed substantial activity. Why this occurred was unknown, but the impurity of the specimen probably contributed to this smearing of activity into adjacent samples. Alternately, more than one stimulant may be present in whole cornea compared with the more purified collagenous tissue.

We found that the stimulant derived from the collagen preparation and whole cornea was a large polypeptide of a noncollagenous type. The amino acid composition from the active HPLC fraction from both samples suggested that the stimulant was a protein. In both cases the active HPLC fraction had negligible hydroxyproline and a low glycine content; therefore it was not collagen. Treatment of SAC with ultrapure bacterial collagenase increased stimulatory activity, additional assurance that the stimulatory factor was not derived from the collagen molecule. These results were compatible with the idea that the respiratory stimulant may, in part, be embedded in the collagen framework and released as the collagen matrix is degraded.

Key words: alkali, collagen, cornea, bacterial collagenase, high-performance liquid chromatography, amino acid composition

Acknowledgments

The authors thank the Texas Agricultural Experiment Station Biotechnology Support Laboratory, College Station, Texas for determining the amino acid composition of the stimulant samples and Terrell James for technical assistance.

References

1. Brown SI and Weller CA: The pathogenesis and treatment of collagen induced diseases of the cornea. Trans Am Acad Ophthalmol Otolaryngol 74:375, 1970.
2. Kenyon KR, Berman M, Rose J, and 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 18:570, 1979.
3. Foster CS, Zelp RP, Mai-Phan T, and Kenyon KR: Immunosuppression and selective inflammatory cell depletion: Studies on a guinea pig model of corneal ulceration after ocular alkali burning. Arch Ophthalmol 100:1820, 1982.
4. Paterson CA, Williams RN, and Parker AV: Characteristics of polymorphonuclear leukocytic infiltration into the alkali burned eye and the influence of sodium citrate. Exp Eye Res 39:701, 1984.
5. Pfister RR and Burnstein N: The alkali burned cornea: I. Epithelial and stromal repair. Exp Eye Res 23:519, 1976.
6. Pfister RR, Haddox JL, Dodson RW, and Harkins LE: Alkali-burned collagen produces a locomotory and metabolic stimulant to neutrophils. Invest Ophthalmol Vis Sci 28:295, 1987.
7. Pfister RR, Haddox JL, Lam K-W, and Lank KM: Preliminary characterization of a polymorphonuclear leukocyte stimulant isolated from alkali-treated collagen. Invest Ophthalmol Vis Sci 29: 955, 1988.
8. Berry SM, Hong BS, Lam K-W, Haddox JL, and Pfister RR: Degradation of bovine corneal collagen by alkali. Cornea 8: 150, 1989.
9. Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193:265, 1951.
10. Ferrante A and Thong YH: Optimal conditions for simultaneous purification of mononuclear and polymorphonuclear leukocytes from human blood by the Hypaque-Ficoll method. J Immunol Methods 36:109, 1980.
11. Pfister RR, Haddox JL, Harkins LE, and Dodson RW: The effects of citrate on fMLP-induced polymorphonuclear leukocyte stimulation and locomotion. Cornea 3:183, 1984/1985.