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Preliminary Characterization of a Polymorphonuclear Leukocyte Stimulant Isolated From Alkali-Treated Collagen

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This study reports the preliminary characterization of a stimulant released from alkali-treated collagen which activates the respiratory burst of PMNs. The supernatant fraction from alkali-treated collagen (SATC) was precipitated with ammonium sulfate, resuspended, and centrifuged through a sucrose gradient (10–30%, W/V). Proteins were detected throughout the gradient but PMN stimulatory activity was found mainly in fractions 1 and 2 (bottom of gradient), indicating a very high molecular weight. When SATC was layered on a denser sucrose gradient (20–85%, W/V), protein was spread over the upper half of the gradient. The peak of PMN stimulatory activity was found in the middle of the gradient (fractions 8 and 9), indicating a molecular weight similar to the 200,000 dalton standard. High pressure liquid chromatography (HPLC) of fractions from this heavier sucrose gradient showed a small peak, followed by a much higher main peak. The small peak, with the highest molecular weight peptide, is responsible for stimulation of the respiratory burst of PMNs (fraction 8 and 9). The source of this stimulant is yet to be determined. Invest Ophthalmol Vis Sci 29:955–962, 1988

Alkali burns of the eye incite the influx of large numbers of polymorphonuclear leukocytes (PMNs) into the cornea shortly after the burn.[1-5] A number of potential mediators have been implicated in inflamed tissues. In the alkali-injured eye, prostaglandins and leukotrienes are found to be associated with the inflammatory process.[6,7] An additional potential mediator for this PMN invasion may, in part, be a breakdown product(s) from alkali-damaged corneal proteins.[8] The supernatant fraction obtained from alkali-treated collagen (SATC) has been shown to contain substances which enhance PMN locomotion and stimulate the PMN respiratory burst in vitro. High concentrations of the stimulant cause PMN lysis with its attendent release of hydrolytic granular enzymes. We have also shown that SATC is probably not involved in mediating PMN adherence to the vascular endothelium, as indicated by the absence of an effect on the adherence of PMNs to nylon fibers in vitro in the presence of serum albumin.[9] On the strength of these data we have suggested that breakdown product(s) from alkali damaged corneal proteins may play a role in the attraction of PMNs into the alkali injured cornea. In the central portion of the cornea it is likely that high concentrations of the stimulant(s) and low levels of albumin activate PMN respiration, leading to superoxide radical production and ultimately their lysis with the release of hydrolytic enzymes.

The purpose of this study was to partially isolate and characterize the stimulant(s) released from alkali-treated collagen which activates the respiratory burst of PMNs.

Materials and Methods

Materials

$CaCl_2$ and ammonium sulfate were purchased from Mallinckrodt (St. Louis, Mo). $MgCl_2$ and sucrose were purchased from Fisher Scientific Co. (Fair Lawn, NJ) and Baker Chemical Co. (Phillipsburg, NJ), respectively. Human serum albumin (HSA), free of fatty acid, and sodium azide were obtained from Sigma Chemical Co. (St. Louis, Mo). Hanks balanced salt solution (HBSS) was obtained from Gibco Laboratories (Grand Island, NY).

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Collagen Preparation

Type 1 collagen was obtained from Sigma Chemical Co. (Cat. no. C-4387, insoluble). Collagen was treated with 1N NaOH (w/v, 1:6). Alkali treatment of collagen was carried out at 35°C for 30 min, mixing thoroughly at 10 min intervals, followed by a 5 to 15 min titration with 1N HCl (approximately 0.9 ml HCl/ml NaOH) to pH 7.4. The neutralized suspension was centrifuged at 15,000 *g* for 10 min to remove particulate material. Sodium azide (0.01%) was added to the resulting supernatant (SATC). Control samples were prepared in an identical manner except Sigma collagen was treated with 0.5 M NaCl (prepared by titrating 1N NaOH with 1N HCl to a pH of 7.4) substituted for 1N NaOH and 1N HCl.

Ammonium Sulfate Precipitation

Ammonium sulfate was added to SATC until 40% saturated. The solution was mixed for 10 min and then subjected to centrifugation at 15,000 *g* for 10 min. The protein precipitated at 40% saturation of ammonium sulfate is designated as ASP_1. The protein precipitated when ammonium sulfate was increased in the supernatant fraction from 40–55% saturation and from 55–65% saturation are designated as ASP_2 and ASP_3 respectively. The precipitated proteins (ASP_1, ASP_2 and ASP_3) were resuspended in water containing 0.01% sodium azide at one-third the original supernatant volume and subjected to a 2 hr centrifugation at 100,000 *g*. The supernatant fractions were dialyzed for 4 hr.

Sucrose Density Gradient

A 0.6 ml aliquot of the above supernatant fraction (ASP_1) was placed on top of 4.8 ml of sucrose density gradient solution A (10–30%, w/v) or B (20–85%, w/v) containing 0.01% sodium azide in a nitrocellulose centrifuge tube. These tubes were placed in a swing bucket rotor (Beckman SW 50.1) and centrifuged at 35,000 rpm for 16 hr at 25°C. Subsequently, a hole was punctured in the bottom of the tube with a 25 gauge needle, and the sucrose gradient was collected into six drops/fraction. These fractions were analyzed for respiratory burst activity and molecular weight by HPLC. The protein concentration of each fraction was analyzed by the method of Lowry et al.[20] The Lowry protein assay underestimates the concentration of collagen. The technique is satisfactory for the objective of this study, which is to locate the active stimulant as it relates to the protein distribution throughout the sucrose gradient. The interference of sucrose in the protein assay is negligible, as can be seen in the early fractions (where there is more sucrose) noted in Figure 6A.

High Pressure Liquid Chromatography (HPLC)

All fractions from sucrose density gradients were analyzed by HPLC. A Protein PAK 300 SW column, supplied by Waters Associates (Milford, MA), was equilibrated in 0.15 M NaCl-10 mM phosphate (pH 7.5) and eluted by the same buffer at a rate of 1 ml/min. A 1 μl sample was injected into the column. The eluate was monitored at 210 nm and 280 nm in a UV monitor (Waters Associate model 490 multiwavelength detector).

PMN Isolation

Following the technique of Ferrante and Thong,[11] PMNs were isolated from fresh human whole blood by centrifugation on Hypaque-Ficoll (density 1.114) as described in a previous paper.[8] Isolated PMNs were resuspended in Hanks' balanced salt solution (HBSS), containing 500 μM Ca^{2+} and 600 μM Mg^{2+}, to a purity of 90.0% ± 1.8% (n = 6) PMNs (96–99% viability) with the remaining percentage consisting of red blood cells (RBCs) and less than 5% platelets, lymphocytes, and eosinophils.

PMN Respiratory Burst

All stock solutions were prepared fresh each day in HBSS or distilled water, both containing 500 μM Ca^{2+} and 600 μM Mg^{2+}, at pH 7.4. All fractions from sucrose density gradient centrifugation were dialyzed in 1000 molecular weight cutoff tubing (Spectrum Medical Industries, Inc., Los Angeles, CA) against distilled water (0.01% sodium azide) for 6 hr (2 liters/2 hr) and 2 liters of HBSS containing sodium azide for overnight.

The respiratory burst of PMNs (4.5×10^6 cells/ml) 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. A 400 μl aliquot of each dialyzed sucrose gradient fraction was mixed with 400 μl of 0.5 M NaCl to enhance the respiratory burst.[8] This mixture was prewarmed and added to 200 μl of prewarmed PMNs in HBSS to make a total volume of 1 ml. Oxygen consumption was measured for 10 min.

When testing the effect of serum albumin on the stimulant from alkali-treated collagen, HSA (1.2 mg/ml) was added 30 seconds after SATC (100 μl/ml incubation mixture).

Ultrafiltration

SATC was centrifuged at 600 *g* for 2 hr in Centriflo ultrafiltration membrane cones (CF 50, Amicon Corporation, Danvers, MA) to concentrate polypeptides greater than 50,000 molecular weight.

Table 1. The effect of alkali- or salt-treated collagen on PMN incubations

	μl supernatant per ml incubation	*mg protein per ml incubation*	*Percent O_2 consumption per min per ml incubation*	*mOs/kg*
Supernatant from NaOH	65	0.438	4.0	322
treated collagen	75	0.506	4.3	325
(6.74 mg/ml)	100	0.674	7.3	381
Supernatant from NaCl	100	0.096	0	358
treated collagen	200	0.192	0	417
(0.96 mg/ml)	400	0.384	0	583
	800	0.768	3.0	827

Statistics

Statistical analysis was performed with the student t-tests. Standard error of the mean is used throughout the paper.

Results

Respiratory Burst Activity of Alkali- or Salt-Treated Collagen

In this study the same amount of collagen (87.7 mg/ml) was suspended in the alkali- or salt-treated samples. The amount of protein solubilized after treatment with NaOH is seven-fold more than that by NaCl. The soluble fraction of the alkali-treated collagen (SATC) and the salt-treated collagen were analyzed for their effect on PMN respiration (Table 1). When the same volume of SATC or salt-treated collagen was added to the incubation mixture, SATC is much more active than salt-treated collagen. Only 100 μl of SATC per milliliter of incubation mixture was required to produce an optimal stimulation on PMN respiration, while no effect was observed up to 400 μl of salt-treated collagen. Salt-treated collagen (800 μl) produced a moderate increase of PMN respiration while its protein concentration in the incubation chamber exceeded that of the volume of SATC (100 μl) needed to obtain optimal stimulation. The respiratory burst by salt-treated collagen was achieved in the presence of extreme hypertonicity caused by the large volume of salt mixed in the incubation chamber.

There were important physical and physiological characteristics of the stimulatory agent(s) noted in the SATC. The stimulant(s) is relatively heat- and cold-stable, although 60°C slightly reduced its stimulatory capacity ($0.001 < P < 0.01$) (Fig. 1). Human serum albumin inhibited the stimulant(s)-induced respiratory burst ($P < 0.001$) and prevented the previously reported development of plasma membrane blebs[8] in PMNs (Fig. 2). Although stimulatory activity was lost after dialysis of sucrose gradient fractions in 1000 molecular weight cutoff tubing, it was regained after the addition of salt back to these samples (see Figs. 5C, 6C). The concentrate from ultrafiltration of SATC retained the respiratory burst activity, while no activity was present in the filtrate (Fig. 3).

Ammonium Sulfate (AS) Fractionation of SATC

Respiratory burst activity was recovered in the protein precipitated by 40% saturation of ammonium sulfate (ASP_1) (Table 2). No activity was noted in the additional proteins precipitated when ammonium sulfate was increased to 55% (ASP_2) and 65% (ASP_3) saturation. Some of the material in ASP_1 could not be redissolved. The insoluble material was removed by centrifugation at 100,000 *g* for 2 hr. Full activity was recovered in the supernatant fraction (Table 3).

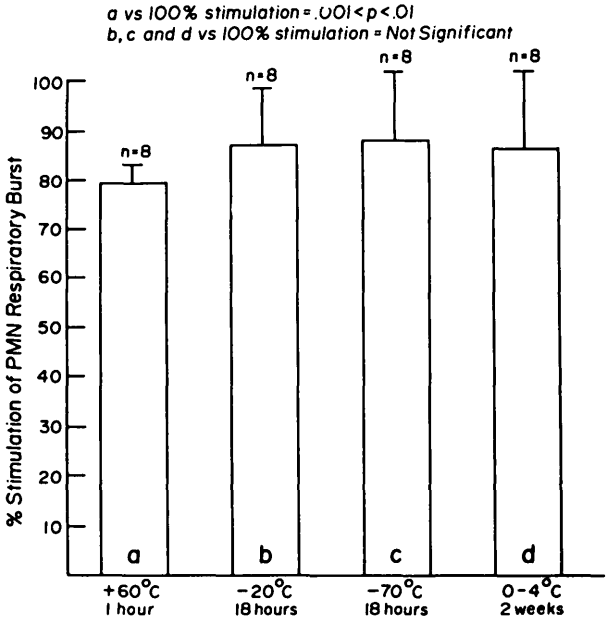

Fig. 1. The stimulatory capacity of SATC is not meaningfully affected by large variations in temperature. Aliquots of SATC were exposed to different temperatures, prewarmed to 37°C, and added to the incubation mixture (100 μl supernatant/ml incubation mixture).

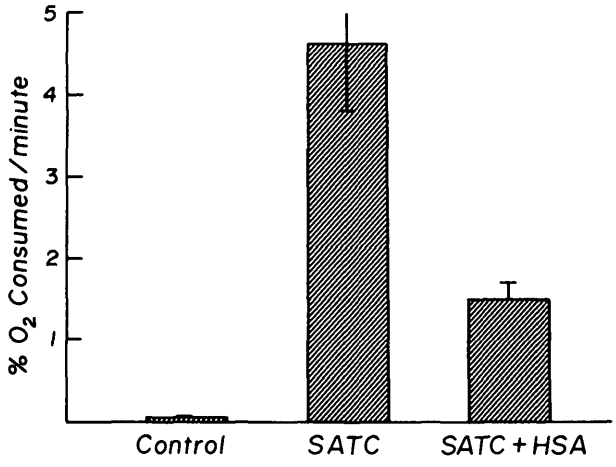

Fig. 2. HSA (1.2 mg/ml) has a significant inhibitory effect ($P < 0.001$) on oxygen consumption by PMNs exposed to SATC. Control incubations contain PMNs only. SATC incubations contain PMNs stimulated by supernatant from alkali-treated collagen. SATC plus HSA incubations contain stimulated PMNs inhibited by HSA. All bars represent the means of five incubations.

HPLC Analysis of ASP_1

HPLC analysis of ASP_1 (subjected to high speed centrifugation) indicated a high degree of heterogeneity (Fig. 4). A sharp peak (I) appeared in the exclusion volume corresponding to 400,000 for native globular proteins and 150,000 for randomly coiled proteins. A broad peak (II) appeared next, indicating the occurrence of peptides with a broad range of molecular

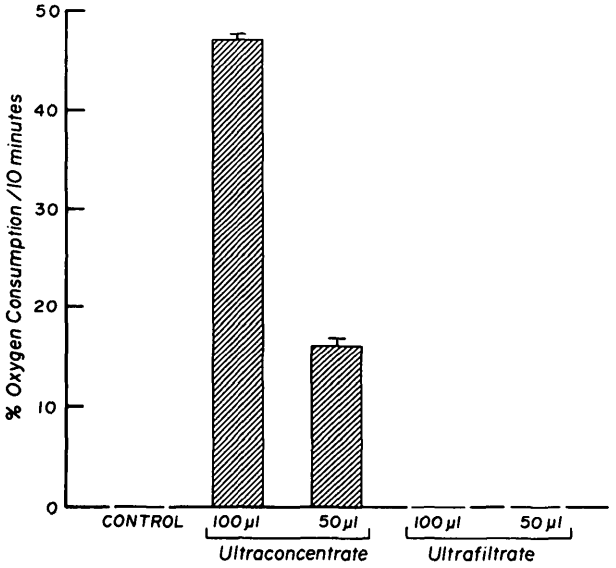

Fig. 3. Oxygen consumption by PMNs exposed to ultrafiltered SATC. Ultrafiltrate or ultraconcentrate (50 or 100 µl) were added per milliliter of incubation mixture. The concentration of peptides greater than 50,000 molecular weight in the ultraconcentrate was twice that of the original SATC. All bars represent the mean of four incubations.

Table 2. Respiratory burst activity in ammonium sulfate (AS) precipitated fractions from SATC

	Sample	*Percent Oxygen Consumption/Min*
*ASP_1	(0–40% saturation)	5.8 ± 1.0, N = 6
ASP_2	(40–55% saturation)	0.0 ± 0.0, N = 5
ASP_3	(55–65% saturation)	0.0 ± 0.0, N = 5

* Pellets were resuspended in HBSS at ⅓ the original volume. Incubation mixtures consisted of 200 µl of resuspended pellet and 800 µl of PMNs in HBSS.

sizes. The eluate from the column was monitored at 210 and 280 nm. Both peaks were marked by low absorption at 280 nm, signifying a low aromatic amino acid content, characteristic of collagen. There was insufficient sample in the eluate to analyze for biological activity.

Sucrose Density Gradient Centrifugation

ASP_1 (subjected to high speed centrifugation) was layered on sucrose density gradients to separate peptides based on their molecular size. Two ranges of sucrose density gradients were used: (1) 10–30%, w/v; and (2) 20–85%, w/v. When gradient 1 was used, proteins were detected throughout the gradient peaking in the middle fractions (Fig. 5A). Analysis of each fraction by HPLC (Fig. 5B) showed a high molecular weight component (peak I) and a broad peak (II) representing low molecular weight peptides. The last peak seen in all fractions is due to the high concentration of sucrose throughout the gradient. Peak I was highest in fractions 7 and 8, while peak II was highest in fractions 9 and 10. However, the respiratory burst activity was observed mainly in fractions 1 through 3 (Fig. 5C), where only small amounts of either peak was observed. When gradient 2 was used, protein was spread over the upper half of the gradient (Fig. 6A). Analysis of fractions from gradient 2 by HPLC shows two high molecular weight peaks (Ia and Ib), with the small one (Ia) appearing just before the main peak (Ib) (Fig. 6B). Ib reached the highest point at fraction 11. The magnitude of Ia is much smaller than Ib, yet

Table 3. Respiratory burst activity in ammonium sulfate (AS) precipitated and ultracentrifuged supernatant

Sample	*Percent Oxygen Consumption/Min*
ASP_1-Concentrated 3×, 45 µl	3.9 ± 0.5, N = 3
*Ultracentrifuged pellet, 45 µl	0.1 ± 0.1, N = 3
Ultracentrifuged supernatant, 45 µl	4.4 ± 0.3, N = 3

* Pellet resuspended in HBSS at the original volume. Incubation mixtures consisted of 45 µl of sample and 955 µl of PMNs in HBSS.

the respiratory burst activity in Ia is higher than Ib (fractions 8 and 9, Fig. 6C). Therefore, Ia is the most potent component.

The molecular weight of Ia was evaluated by sucrose density gradients (SDG) and by HPLC. Sucrose density gradients separate peptides based on their density. On SDG, Ia peaked in the area of amylase which is a molecular weight marker for 200,000 daltons (Fig. 7). HPLC separates peptides based on size. Ia appeared in the exclusion volume of HPLC (column PAK 300) which excludes all peptides greater than 400,000 daltons for native globular protein and 150,000 daltons for randomly coiled proteins (Fig. 6B).

Discussion

The results of this study show that the stimulatory agent in SATC appears to be a large macromolecule (about 200,000 daltons). This size determination is based on the distribution of activity in the middle (20–85%) and bottom (10–30%) fractions of the sucrose density gradients, respectively. Lipid impurities (<0.1%), fractionating into the top portion of both gradients, were not stimulatory. Although chemoattractive agents have been identified in the range of 100,000 molecular weight,[12] the vast majority are of considerably smaller size. The large size of the respiratory burst stimulant in this paper suggests that if it is present in the alkali-injured cornea it mediates only the metabolic stimulation of PMNs in these corneas and not their chemoattraction.

Respiratory burst activity is lost when the stimulatory fractions are dialyzed in 1000 molecular weight cutoff tubing. If the osmolalities of these fractions are then raised to hypertonic levels with NaCl the activity is restored. We have previously reported this finding and concluded that dialysis does not induce a loss of stimulant(s), but that hypertonicity enhances the stimulatory effect.[8] This conclusion is supported by the results showing that ultrafiltration concentrated the stimulant(s) with no activity present in the filtrate (<50,000 daltons). This enhancing effect of hypertonicity may have played a role in the activation of the respiratory burst of PMNs exposed to NaCl-treated collagen.

Since NaCl treatment cannot break peptide bonds, activation of the respiratory burst of PMNs by extremely large volumes of the supernatant fraction from NaCl-treated collagen indicates that the stimulant(s) could be the entire solubilized collagen molecule, with some modified conformation, or other macromolecule. Alkali treatment appears to solubilize significantly more collagen than NaCl, and to enhance the stimulatory process. This is evident from the finding that alkali-treated collagen solubilizes

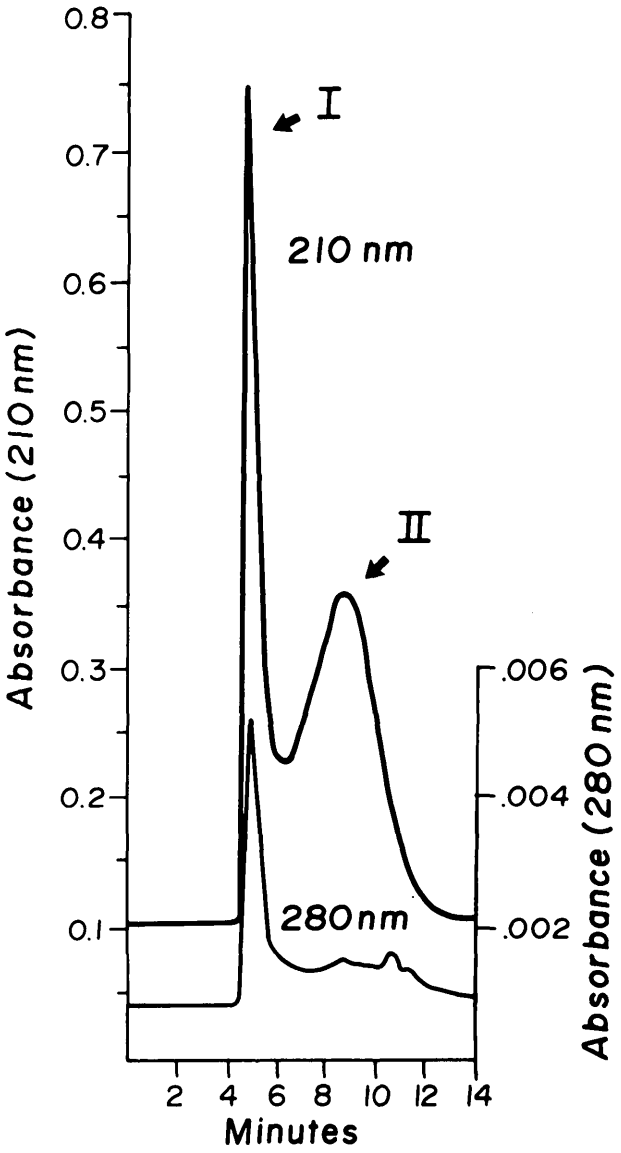

Fig. 4. High pressure liquid chromatography of degradation products of alkali-treated collagen precipitated by ammonium sulfate. Detection of peptides, based on the absorbance of aromatic amino acids at 280 nm, showed a very minor peak at 5.5 min, the void volume of the column. The detection of peptides at 210 nm showed a sharp peak at 5.5 min, followed by a broad peak, the latter indicating the heterogenous nature and low aromatic amino acid content of the small peptides. Note the two different ordinates for the 210 nm and 280 nm absorbance on the right and left scales, respectively.

about seven times more protein than salt-treated collagen and that about eight times more volume of salt extract is required to produce a detectable respiratory response, even with the enhancing effect of a high osmolality.

Alkali treatment involves partial cleavage of the collagen molecule, possibly exposing more stimulatory sites. This is shown by the large amount of heterogeneous peptides present in the sucrose density gra-

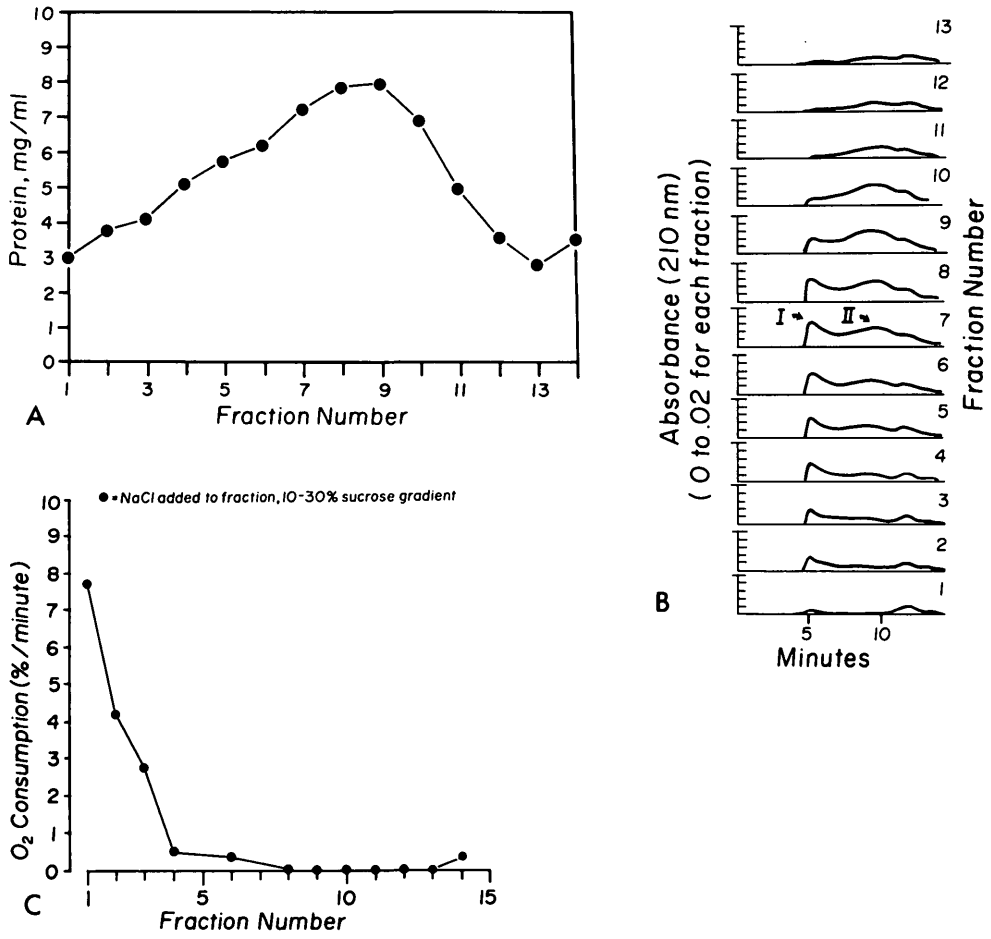

Fig. 5. Analysis of alkali-treated collagen fractions after sucrose density gradient centrifugation (10–30%, W/V). **(A)** The protein concentration (mg/ml) of each fraction was analyzed by the method of Lowry et al.[10] **(B)** The fractions were analyzed by high pressure liquid chromatography. The bottom fractions mainly contain the high molecular weight product (peak I) while the small peptides were seen in the upper fractions (peak II). **(C)** The fractions (400 μl) were dialyzed and then assayed for their stimulatory activity by incubation with PMNs in HBSS (200 μl) and an additional 400 μl of 0.5 M NaCl. The consumption of oxygen was recorded for 10 min.

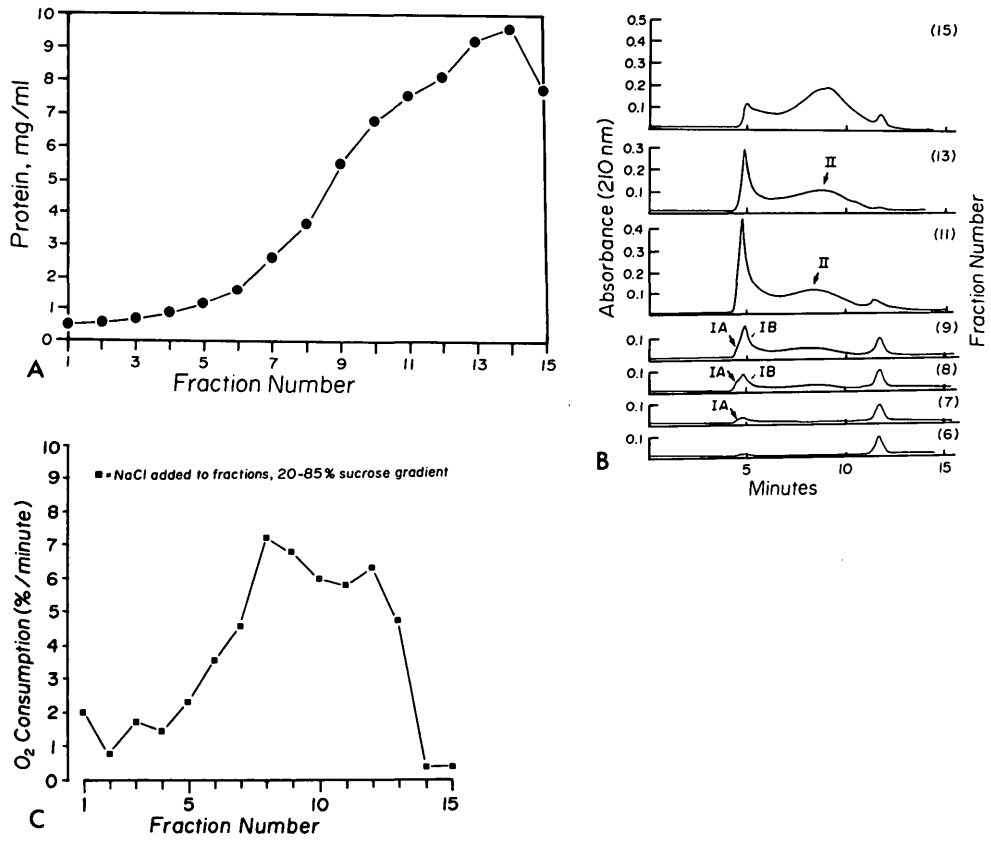

Fig. 6. Analysis of alkali-treated collagen fractions after sucrose density gradient centrifugation (20–85%, W/V). **(A)** The protein concentration (mg/ml) of each fraction was analyzed by the method of Lowry et al.[10] **(B)** The fractions were analyzed by high pressure liquid chromatography. The appearance of a double peak of high molecular weight peptides was noticed in fractions 7–9. The highest concentration of small peptides occurred in fraction 15. **(C)** The fractions (400 μl) were dialyzed and then assayed for their stimulation activity by incubation with PMNs in HBSS (200 μl) and an additional 400 μl of 0.5 M NaCl. The consumption of oxygen was recorded for 10 min.

dients and in the eluate after HPLC of SATC. Among the heterogenous degradation products it is the largest fragment which shows stimulatory activity. It should also be noted that a neutral salt extraction of the collagen monomer (tropocollagen) has been shown in the ground substance prior to incorporation in the collagen fibrils.[13] It is possible that the stimulant is partly from fibrillary collagen but also from these prefibrillary collagen subunits in the ground substance prior to their incorporation into fibrils.

Our previous work has shown that a respiratory burst stimulant of PMNs is obtained from alkali-treated cornea or collagen.[8] The evidence presented in this paper indicates that the molecular weight of the stimulant is similar to native collagen. The cornea is mainly type I collagen, identical to the collagen obtained from bovine achilles tendon used in these experiments. We elected to use commercially prepared type I collagen rather than whole cornea, which contains highly heterogeneous cellular macromolecules.

A small amount of glycosaminoglycans (GAGS) is often present in collagen preparations. The present data have not ruled out a possible stimulatory effect from GAGS present in the collagen preparation, but the low concentration of GAGS in our collagen preparation make such a possibility unlikely. In either event the presence of a stimulatory agent of this type is an important finding and has far-reaching implications in the alkali-injured eye.

PMN infiltration occurs as a consequence of the release of inflammatory mediators. There are potent chemoattractants found throughout the body, such as complement components (C3a and C5a[14]) and complexes of superoxide radicals, lipid, and albumin.[15] After alkali injury, other inflammatory mediators have been identified in high concentration in the aqueous humor, including prostaglandins and leukotrienes.[6,7] Plasminogen activator, a known chemotactic agent, is produced in cultures of ulcerating corneas, presumably by the epithelium, PMNs and fibroblasts.[16] Alkali treatment of collagen or corneas releases factors which activate PMN functions in vitro[8] and may be the mediators present in the early time period after alkali injury to the cornea. The large protein fragment studied here might mediate the metabolic stimulation of PMNs, while the previously discovered agent(s) enhances PMN locomotion in the early phase after an alkali injury.

Albumin might play an important role in the inflammatory process, developing in the cornea of the alkali-injured eye. In a previous study we showed that SATC activates PMN locomotion in the presence of serum albumin, possibly by affecting cell-to-substrate adhesion,[17] and activates the respiratory burst in the absence of albumin.[8] This is consistent with our data

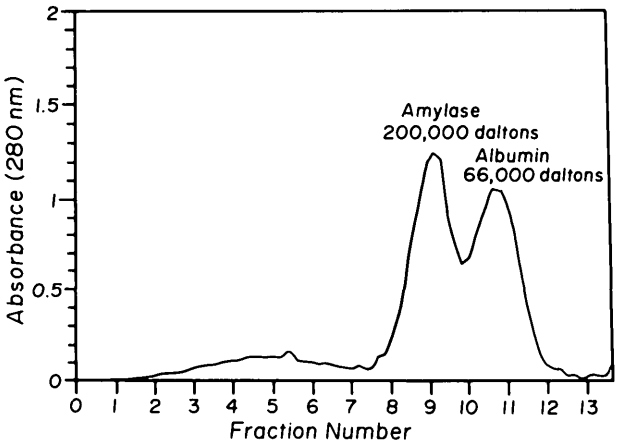

Fig. 7. Macromolecules were separated by sucrose density gradient. A mixture of albumin (66,000 daltons) and amylase (200,000 daltons) was layered on a 20–85% (W/V) sucrose density gradient. Protein separation was determined by measuring the absorbance at 280 nm.

showing that albumin inhibited the respiratory burst generated by SATC. Higher concentrations or prolonged exposure of PMN to SATC in the absence of albumin were also previously reported to cause an increased respiratory burst and trigger PMN lysis, releasing lysosomal enzymes.[8] In another study, we showed that serum albumin protects PMNs from lysis during prolonged exposure to SATC.[9] In an independent study, Boisjoly showed that eye drops containing albumin significantly reduced the loss of stromal thickness after severe alkali injury of the rabbit eye.[18] It was suggested that albumin acted as a nonspecific enhancer of epithelial regrowth, similar to fibronectin. Alternately, albumin might have prevented ulceration by inhibiting the respiratory burst and preventing lysis of PMNs.

The present findings and our earlier report lead us to propose a new hypothesis of inflammatory mediation developing soon after alkali injury of the cornea (Fig. 8). In this hypothesis the concentration of albumin is critical to the inflammatory cell response in the cornea. Specific albumin levels in burned corneas are not known, but it has been shown that there is a seven-fold drop in concentration from normal limbal blood vessels to the peripheral cornea and a further three-fold drop from peripheral to central cornea.[19] In the alkali-burned eye, PMNs extravasating from patent limbal or paralimbal vessels could be chemotactically drawn into the peripheral cornea by a locomotory agent(s) from alkali-injured collagen. High levels of albumin near the limbus would facilitate PMN locomotion while blocking the stimulatory capacity and potentially lytic effect of the large collagen-derived polypeptide which activates the PMN respiratory burst (Fig. 8—1). As the PMNs locomote from the peripheral into the central corneal tissue,

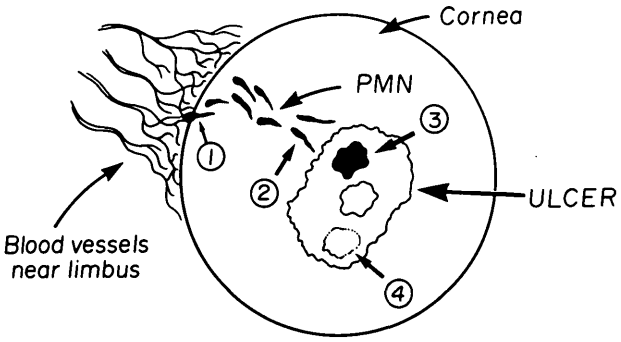

Fig. 8. A hypothesis suggesting that collagen breakdown product(s) are inflammatory mediators in the alkali injured eye. (1) Locomotory agent(s) from alkali-injured collagen might cause locomotion of PMNs into the peripheral cornea in the presence of serum albumin. (2) As PMNs approach the central cornea their locomotion decreases because of reduced albumin levels. (3) High levels of the stimulant from alkali-injured collagen induces the respiratory burst and superoxide radical production of PMNs in the relative absence of albumin. (4) PMN lysis occurs, triggering the release of granules containing hydrolytic enzymes.

they might be exposed to a significant further reduction of serum albumin. This lowered concentration of albumin in the central cornea might reduce the locomotory stimulus (Fig. 8—2). This reduced albumin level, in addition to much higher levels of the stimulant from alkali injured collagen, would promote the respiratory burst and the release of superoxide radicals in the central portion of the burned cornea[8] (Fig. 8—3). When the stimulant levels are sufficiently high, PMN lysis could be triggered, with the release of granules containing hydrolytic enzymes[8] (Fig. 8—4). In the presence of superoxide radicals and a variety of hydrolytic enzymes, alkali-injured and normal collagen could be damaged further, leading to collagen loss manifested as ulcer formation. Whether these collagen degradation products derived from PMN activities are stimulatory to the PMN respiratory burst or to locomotion is unknown. It is quite likely, however, that the release of inflammatory mediators from PMNs in the injured cornea do recruit further PMNs.

Key words: collagen, glycosaminoglycans, alkali, stimulant, polymorphonuclear leukocytes, respiratory burst

Acknowledgment

Mark Siemans performed many of the biochemical analyses.

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