Effect of Synthetic Metalloproteinase Inhibitor or Citrate on Neutrophil Chemotaxis and the Respiratory Burst

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**Purpose.** A topical synthetic metalloproteinase inhibitor (SIMP) has been reported to reduce the incidence of corneal ulcerations in the alkali-injured eye by the putative mechanism of collagenase inhibition. The current study was performed to determine whether SIMP has a direct inhibitory effect on the activation of neutrophils and to compare any such effect with that of citrate, a known inhibitor of polymorphonuclear leukocyte (PMNL) activity.

**Methods.** The effect of SIMP or citrate was tested on chemotaxis of PMNL and the respiratory burst in vitro.

**Results.** Synthetic metalloproteinase inhibitor (1 mM) or citrate (12 mM) produced significant inhibition of chemotaxis of PMNL and the accompanying behavioral characteristics of motility. Synthetic metalloproteinase inhibitor, but not citrate, generated a dramatic respiratory burst when incubated with resting PMNL. Both SIMP and citrate inhibited the respiratory burst of PMNL in the presence of opsonized zymosan. For a brief, initial period each substance enhanced the respiratory burst generated by the metabolic stimulant from alkali-degraded corneas, followed by a rapid decline in activity that was associated with cell death.

**Conclusions.** Although SIMP has been shown to exhibit powerful collagenase inhibition, its inhibitory effect on chemotaxis of PMNL might be the primary mechanism in reducing the incidence of ulceration in the alkali-injured cornea. Very significant reduction in the accumulation of PMNL in SIMP–treated corneas supports this conviction. Activation by SIMP of the respiratory burst in resting PMNL is of concern, but overall its beneficial effect is favorable, as demonstrated in prior alkali-injured animal models. The dual effect of inhibition of chemotaxis of PMNL and activity of collagenase makes SIMP a potential drug for combating ulceration in the alkali-injured cornea. Invest Ophthalmol Vis Sci. 1997;38:1340–1349.

Polymorphonuclear leukocytes (PMNL) rapidly invade injured tissues in response to the release of chemoattractants from the damaged organ. Local vascular endothelial cells are activated and become sticky for marginating neutrophils. By diapedesis, adherent PMNL move through the vessel wall toward the source of the chemoactivator. The impact of the accumulation of PMNL in corneal tissue is destructive, allowing the release of a variety of hydrolytic enzymes and oxygen free radicals designed to degrade damaged tissues. The destructive activities of PMNL are a major contributor to the development of ulcers in the alkali-injured cornea.1–5

Synthetic metalloproteinase inhibitor (SIMP), a thiol peptide (HSCH₂-Leu-Phe-Ala-NH₂), has been reported to inhibit matrix metalloproteinases, including collagenase in vitro.6–7 It also reduces corneal ulceration after alkali injury to the rabbit eye.8,9 Histopathology of the alkali-injured control eyes showed a heavy infiltration of PMNL into the cornea, but corneas treated with SIMP showed very few PMNL. It was concluded that the major mechanism of action for SIMP was probably its anticollagenase activity.

The purpose of the current study is to determine whether SIMP has a direct inhibitory effect on PMN functions, explaining the absence of PMNL in the corneas treated with SIMP. In addition SIMP will be compared with a known PMN inhibitor, citrate.10,11 The findings will add to the existing base of knowledge on the mechanism of action of SIMP.

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METHODS

Materials

Hanks balanced salt solution (HBSS) was purchased from Gibco Laboratories (Chagrin Falls, OH). Calcium chloride, magnesium chloride, sodium chloride, trisodium citrate, zymosan, glutaraldehyde, Ficoll (Type 400), dimethyl sulfoxide (DMSO) and sodium azide were purchased from Sigma Chemical (St. Louis, MO); sodium hydroxide was obtained from Fisher Scientific (Fair Lawn, NJ); hydrochloric acid was obtained from U.S. Industrial Chemicals Co. (Tuscola, IL); and Vitrogen 100 was purchased from Celsisra Laboratories (Palo Alto, CA).

Frozen bovine eyes were purchased from Pel-Freez Biologicals (Rogers, AR). All experimental procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Preparation of Solutions

All HBSS solutions contained 500 μM Ca²⁺ and 600 μM Mg²⁺. Suspended in methanol solution, LTB₄ was diluted with HBSS to a pH of 7.3 and a final methanol concentration of 0.05% to 0.16%; SIMP was dissolved in acetic acid (final concentration, 0.06% to 0.24%) and diluted with HBSS, pH 7.3. Citrate was dissolved in HBSS, pH 7.3. Ethanol was mixed with HBSS to a pH of 7.3. 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 osmolality was adjusted to between 270 and 320 mOsm and mixed. The sample was then incubated at 35°C for 24 hours. The sample was neutralized by titration with 1N HCl to pH 7.4 and then 0.05% sodium azide was added. The crude suspension was centrifuged at 15,000 g for 15 minutes and the supernatant fraction collected.

The supernatant fraction was then placed in a 30,000 MWt cutoff centriprep system (Amicon, Beverly, MA) and centrifuged at 1,500 g for 30 minutes. The ultraconcentrate was titrated with acetic acid to pH 5 and centrifuged at 15,000 g for 5 minutes. The resulting pellet was extracted with 100% acetonitrile, sonicated for 30 minutes, centrifuged at 15,000 g 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. This sample was used as the undiluted respiratory-burst stimulant from alkali-degraded corneas to measure the PMN respiratory burst.

The ultrafiltrate from the 30,000 MWt cutoff centriprep system was placed into a 3000 MWt cutoff centriprep system (Amicon, Beverly, MA) and centrifuged for 30 minutes at 2500 g. The resulting filtrate was then centrifuged through an Amicon MPS-1 micropartition system with a YM1 Diaflow membrane (1000 MWt cutoff). Each ml of this final ultrafiltrate was dialyzed (Spectra/Por CE, 100 MWt cutoff, Spectrum Medical, Los Angeles, CA) in 500 ml of distilled water for 4 hours, changing the water once per hour. The final molecular size of this ultrafiltrate was between 100 and 1000 MWt. The pH and osmolality were measured and adjusted by adding varying amounts of HBSS or distilled water to a pH of 7.3 and an osmolality of 280 to 320 mOsm. This sample was used as the undiluted chemoattractant from alkali-degraded corneas (CADC) to measure PMN polarization and chemotaxis.

Neutrophil Isolation

These experiments followed the tenets of the Declaration of Helsinki and were approved by the human research committee at Brookwood Medical Center. All donors signed written consent forms explaining the nature and possible consequences of the study. Blood was collected from only one donor each day. Following the technique of Ferrante and Thong,12 PMNL were isolated from fresh, heparinized human blood by centrifuging on a mixture of Hypaque and Ficoll (density = 1.114) according to a previous report.13 Isolated PMNL (96% to 99% viability) were resuspended in HBSS at room temperature and gently agitated on a shaker. The purity of this cell suspension was >85% PMNL, <5% mononuclear cells and platelets, with the remaining percentage consisting of red blood cells. Purified PMNL were used in the polariza-
tion assay, collagen gel–visual chemotactic assay, and the respiratory burst assay to measure their response to SIMP and citrate. All incubation mixtures from these assays maintained an osmolality between 270 and 330, a pH range of 7.2 to 7.6, and a Ca²⁺ and Mg²⁺ concentration of 500 μM and 600 μM, respectively.

**Polarization Assay**

The polarization assay was performed in a double-blind fashion. This assay was used to measure the PMN response to LTB₄ or CADC by quantitating the change in cell shape after exposure. The optimal doses for the chemoattractants, 30 nM LTB₄ and CADC (1:5 CADC:HBSS) were chosen from preliminary dose-response studies. Briefly, 1 × 10⁶ PMNL were suspended in HBSS and exposed to citrate, SIMP, or ethanol in the presence of each chemoattractant. The samples were then stirred in a reaction chamber (total volume = 250 μl) at 35°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. The remaining volume of each suspension was immediately centrifuged at 15,000g for 5 seconds to remove cells. The resulting supernatant was analyzed for lactate dehydrogenase activity. All incubations generated lactate dehydrogenase (LDH) activity correlating with <5% cell death. In each sample, PMNL 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 width X 2). The scores of 100 PMNL for each sample were added, producing a total score that was converted to a polarization index by subtracting the negative control values (PMNL in HBSS only). In most cases, polarization activity was expressed as percentage of inhibition of the positive control.

**Collagen Gel–Visual Chemotactic Assay**

The collagen gel–visual chemotactic assay (CG–VCA) was performed in a double-blind fashion and was used as the conclusive test for the presence of PMN chemotactic activity. The PMN response to LTB₄ or CADC was measured. The optimal doses, 500 nM LTB₄ and undiluted CADC, were chosen from preliminary dose–response studies.

In this assay, the chemotactic movement of PMNL through a collagen gel in response to the chemoattractants was measured directly by a computer-assisted visual tracking system. Immediately before addition to the CG–VCA, the PMN suspension was centrifuged at 60g for 4 minutes. The collagen solution, either plain or containing SIMP, vehicle control (0.24% acetic acid), or citrate, was simultaneously brought to room temperature. The PMN pellet was gently resuspended in the collagen solution (2 × 10⁷ PMN/ml). Four microliters were then added to the system and allowed to gel. Thick-walled dialysis membrane (12,000 to 14,000 MWt cut-off pore size, Spectra/Por 4, Spectrum Medical Industries, Houston, TX) served as a semipermeable barrier between the collagen gel with PMNL and the glass capillary tube containing the chemoattractant.

The microscope was focused at the midpoint of the collagen gel using a micrometer scale on the focusing adjustment of the microscope. All cells in the field of view, clearly visible and identifiable as PMNL, were entered into the study. Movement of PMNL was characterized by analyzing the x and y coordinates of each cell at 30-second intervals. The change in cell position from one time interval to the next was computed as follows:

\[
(\Delta x = x_n - x_{n-1}) \text{ and } (\Delta y = y_n - y_{n-1}),
\]

(both values are in micrometers). Based on these calculations, the following cell behavioral characteristics were derived: displacement for a time interval,

\[
(D = \sqrt{\Delta x^2 + \Delta y^2}),
\]

resultant vector length,

\[
(RVL = \sqrt{(x_n - x_{n-1})^2 + (y_n - y_{n-1})^2}),
\]

velocity, \( V = \frac{D}{\text{min}} \); and the angle of displacement with respect to the x-axis

\[
(\text{Dis} \, \Theta = \tan^{-1} \left[ \frac{\Delta y}{\Delta x} \right]).
\]

The reference angle, derived from the displacement angle, equals 0 when the displacement is in a straight line toward the chemoattractant. The reference angle was measured from time zero to each time interval. Percentage of motility and stop frequency (percentage of motile phase cell that was stopped) were also calculated from the data.

To analyze directional movement further, the chemotactic index of motile PMNL incorporates the resultant vector length times the cosine of the reference angle divided by the total displacement:

\[
\left( \frac{\cos (\text{Ref} \, \Theta) \, RVL}{\Sigma \, D} \right).
\]
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FIGURE 1. Effect of inhibitors on polymorphonuclear polarization. (A) citrate (12 mM) inhibited the polarization of polymorphonuclear leukocytes exposed to 30 nM leukotriene B4 (LTP) or a 1:5 dilution of chemoattractant from alkali-degraded corneas (C). Each data point represents the mean ± SEM from four incubations of polymorphonuclear leukocytes. (B) ethanol (0.25% to 2%) produced an increasing inhibition of polarization of polymorphonuclear leukocytes exposed to 30 nM leukotriene B4 (LTP) or a 1:5 dilution of chemoattractant from alkali-degraded corneas (C). The polarization index for the positive controls, leukotriene B4 and the chemoattractant from alkali-degraded corneas, was 69.5% and 66.5%, respectively.

The relative numbers derived from this formula are an index of the straightness of the PMN path toward the chemoattractant. A positive chemotactic index measures cell movement in the direction of the chemoattractant (+1 = straight line toward the chemoattractant, —1 = straight line directly away from the chemoattractant, and 0 = either a nonmotile PMN or a PMN moving at a 90° angle from the chemoattractant). Cells that moved and then stopped were assigned a chemotactic index for the nonmotile time interval equal to the index immediately preceding the nonmotile phase.

Respiratory Burst

A Flyte 400 luminometer from 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. These free 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 × 10⁶ PMNL were suspended in HBSS, containing luminol (0.2 mg/ml) and DMSO (0.0025%), and exposed to SIMP or citrate in the presence of each respiratory-burst

FIGURE 2. The inhibitory effect of 1 mM synthetic metalloprotease inhibitor (SIMP) on the chemotactic activity of polymorphonuclear leukocytes exposed to (A) 500 nM leukotriene B4 or (B) the undiluted chemoattractant from alkali-degraded corneas. Inhibition becomes less obvious in the final minutes as the chemotactic gradient decays, decreasing the chemotactic index for polymorphonuclear leukocytes in the vehicle control. Each data point represents the chemotactic index (mean ± SEM) at each minute for all polymorphonuclear leukocytes. The solid circles represent the vehicle control gel (A) 10⁴ polymorphonuclear leukocytes and (B) 75 polymorphonuclear leukocytes. The solid squares represent the SIMP gel (A) 10⁶ polymorphonuclear leukocytes and (B) 76 polymorphonuclear leukocytes. A positive chemotactic index of +1 = straight line toward chemoattractant.
TABLE 1. Effect of 1 mM SIMP on the Behavioral Characteristics of PMN Chemotaxis (0 to 10 minutes)

<table>
<thead>
<tr>
<th></th>
<th>Motility (%)</th>
<th>Stop Frequency (%)</th>
<th>Velocity (µm/minute)</th>
<th>Displacement (µm)</th>
<th>Resultant Vector Length (µm)</th>
<th>Duration of Motility (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 nM LTB4</td>
<td>72.1</td>
<td>17.3 ± 1.8</td>
<td>9.7 ± 0.4</td>
<td>90.9 ± 4.8</td>
<td>34.9 ± 2.8</td>
<td>9.4 ± 0.1</td>
</tr>
<tr>
<td>SIMP (106 motile PMNL)</td>
<td>36.8</td>
<td>50.6 ± 2.9</td>
<td>3.7 ± 0.2</td>
<td>24.9 ± 1.9</td>
<td>8.9 ± 0.5</td>
<td>8.0 ± 0.3</td>
</tr>
<tr>
<td>Undiluted CADC</td>
<td>Control (75 motile PMNL)</td>
<td>74.7</td>
<td>27.5 ± 2.3</td>
<td>6.9 ± 0.4</td>
<td>56.1 ± 4.0</td>
<td>26.4 ± 2.3</td>
</tr>
<tr>
<td>SIMP (76 motile PMNL)</td>
<td>55.5</td>
<td>42.2 ± 3.0</td>
<td>4.2 ± 0.2</td>
<td>28.4 ± 1.8</td>
<td>13.3 ± 1.5</td>
<td>7.7 ± 0.2</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. Behavioral characteristics of experimental samples were significantly different (P < 0.001) from controls, except for duration of motility, which was not significant. SIMP inhibited baseline motility for 5 minutes before the introduction of both chemoattractants at time zero: control = 18.3%; SIMP = 0%; control 10.7%; SIMP = 0%.

SIMP = synthetic metalloproteinase inhibitor; PMNL = polymorphonuclear leukocyte.

stimulant. The samples were incubated in a lumino-meter reaction chamber (total volume, 250 µl) at 35°C for 10 to 20 minutes. This assay was performed in a double-blind fashion.

Lactic Dehydrogenase Assay

To evaluate cell viability, the activity of LDH was measured in the extracellular supernatant fraction after centrifugation (15,000g for 1 minute) at the end of each incubation sample. Lactic dehydrogenase release was expressed as a percentage of the total LDH released by Triton X-100 (0.2%) for 30 minutes at 37°C. The activity of LDH was compared with trypan blue staining of the cellular fraction, demonstrating a close correlation.

Statistics

Experiments were performed to determine the inhibitory effect of each test substance on activation of PMNL. Student's t test was used to analyze the data from polymorphonuclear polarization experiments. In the chemotaxis experiments, a population of cells was defined as the number of tracked cells in three separate collagen gels exposed to the same sample. A randomized balance—incorporate block design of the Dunnett's test for analysis of variance was used to evaluate the chemotactic indices of test samples for specific differences from the control. The behavioral characteristics of motile PMNL exposed to the chemoattractants in test and control samples was analyzed for significance by Student's t test.

RESULTS

Polarization

The chemoattractant from alkali-degraded corneas and LTB4 induced a change in the shape of PMNL from round resting cells with a smooth membrane to stretched, irregularly shaped, polarized cells. Pretreatment with SIMP did not significantly prevent this polarization response to LTB4 (165 μM SIMP = 2.4% inhibition, 330 μM SIMP = 4.5% inhibition, 660 μM SIMP = 6.6% inhibition) or CADC (165 μM SIMP = 0% inhibition, 330 μM SIMP = 0% inhibition, 660 μM SIMP = 0% inhibition). The SIMP (0.5 mM) or the SIMP vehicle (0.12% acetic acid in HBSS) alone significantly inhibited (P < 0.001) polarization index in resting PMNL of 70% ± 12% (n = 4) and 42% ± 14% (n = 4), respectively. In contrast, 12 mM citrate significantly inhibited (P < 0.001) the activation of PMN polarization by approximately 45% (Fig. 1A) but had no effect on resting PMNL. The presence of ethanol (0.25% to 2%) in the incubations of PMNL strongly inhibited the activation of polarization (Fig. 1B).

Chemotaxis

Synthetic metalloproteinase inhibitor (1 mM) generated a significant inhibition (0.001 < P < 0.01) of LTB4—induced neutrophil chemotaxis (Fig. 2A). A more extensive elaboration of the motile behavior of PMNL exposed to this chemoattractant is found in Table 1. The percentage of motile PMNL was reduced from the same values in control cells. Only one half of the cells never moved, stop frequency increased significantly from 17% to 51%. The velocity, displacement, and resultant vector length were all significantly reduced from the same values in control cells. Only the duration of motility remained the same for those cells that were motile. Before the introduction of LTB4, SIMP inhibited motility to zero.

Synthetic metalloproteinase inhibitor (1 mM) significantly inhibited (P < 0.05) chemotaxis of PMNL activated by CADC (Fig. 2B). Table 1 demonstrates a significant inhibition on the motile behavior of these cells, except for the duration of motility. One half of the cells never moved, stop frequency increased from 29.5% to 42.2%, and the velocity of motile cells decreased from 7 µm/min to 4.2 µm/min. The displace-
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ment and resultant vector length were reduced by 75%; SIMP reduced motility to zero before the introduction of CADC.

Citrate (12 mM) significantly inhibited chemotaxis of PMNL induced by LTB4 (Fig. 3A, 0.02 < P < 0.05) or CADC (Fig. 3B; P < 0.05). Table 2 demonstrates the capability of citrate to reduce the motile behavior of PMNL. Except for the duration of motility, each motile characteristic of citrate treated PMNL exposed to LTB4 was reduced significantly below levels in control cells. All motile characteristics of citrate-treated PMNL exposed to CADC were significantly reduced below levels in control cells. Before the introduction of either chemotactant, the number of motile PMNL in the citrate-treated gels was reduced significantly when compared to that in with control gels.

Respiratory Burst

Synthetic metalloproteinase inhibitor stimulated a significant respiratory burst in resting PMNL, increasing as the concentration of SIMP decreased from 1 mM to 0.25 mM (Fig. 4). Incubations of PMNL exposed to 1 mM SIMP exhibited <5% frequency of cell death as measured by the LDH assay.

The respiratory burst of opsonized zymosan–stimulated PMNL was dramatically inhibited by SIMP at almost all time points at 1 mM whereas at 0.75 mM the burst of PMNL was inhibited only after 10 minutes (Fig. 5A). Lower concentrations of SIMP (0.50 mM and 0.25 mM) exhibited a moderate stimulation of PMNL. Incubations of PMNL exposed to 1 mM SIMP exhibited <5% frequency of cell death, measured by the LDH assay.

The respiratory burst of PMNL activated by the metabolic stimulant from alkali-degraded corneas is dramatically enhanced by SIMP, above that in the positive control cells, during the period of 3 to 5 minutes, decreasing as time passes (Fig. 5B). The higher the concentration of SIMP, the faster this stimulation was lost, falling to baseline levels. The loss of respiratory burst activity in these cells correlated with the release of LDH and cell death. Incubations of PMNL exposed to the respiratory burst stimulant alone exhibited a 13.1% frequency of cell death, measured by the LDH assay. Cell death increased to 37.7% in the presence of both vehicle control and the respiratory burst stimulant and increased to 100% in the presence of both 1 mM SIMP and the respiratory-burst stimulant.

Citrate strongly inhibited the respiratory burst in PMNL generated by opsonized zymosan, in a manner consistent with the increase of inhibition from the lowest to the highest concentration (Fig. 6A). Incubations of PMNL exposed to 12 mM citrate exhibited <5% frequency of cell death, measured by the LDH assay.

Overall there was a mixed effect of citrate on the respiratory burst in PMNL generated by the metabolic stimulant from alkali-degraded cornea (Fig. 6B). In short periods, 0.5 minutes to 3 minutes, citrate actually enhanced the respiratory burst activated by alkali-degraded corneas, but subsequently a rapid decrease in activity occurred. This enhancing effect was much more prominent with the lowest concentration of citrate and greatly reduced at the highest concentrations. The loss of respiratory-burst activity in these cells
TABLE 2. Effect of 12 mM Citrate on Behavioral Characteristics of PMN Chemotaxis (0 to 10 minutes)

<table>
<thead>
<tr>
<th></th>
<th>Motility (%)</th>
<th>Stop Frequency (%)</th>
<th>Velocity (µm/minute)</th>
<th>Displacement (µm)</th>
<th>Resultant Vector Length (µm)</th>
<th>Duration of Motility (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 nM LTB4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (156 motile PMNL)</td>
<td>92.9</td>
<td>7.2 ± 0.9</td>
<td>11.3 ± 0.2</td>
<td>108.7 ± 2.6</td>
<td>55.0 ± 2.4</td>
<td>9.3 ± 0.1</td>
</tr>
<tr>
<td>Citrate (129 motile PMNL)</td>
<td>82.2</td>
<td>14.9 ± 1.3</td>
<td>9.4 ± 0.3</td>
<td>86.0 ± 3.1</td>
<td>47.2 ± 2.6</td>
<td>9.1 ± 0.1</td>
</tr>
<tr>
<td>Undiluted CADC Control (124 motile PMNL)</td>
<td>88.1</td>
<td>13.2 ± 1.0</td>
<td>9.3 ± 0.3</td>
<td>83.8 ± 2.7</td>
<td>35.5 ± 1.8</td>
<td>8.9 ± 0.2</td>
</tr>
<tr>
<td>Citrate (144 motile PMNL)</td>
<td>55.6</td>
<td>26.7 ± 1.6</td>
<td>6.3 ± 0.1</td>
<td>45.1 ± 2.5</td>
<td>21.8 ± 1.3</td>
<td>7.5 ± 0.2</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. Behavioral characteristics of experimental samples were significantly different (P < 0.05 for LTB4 and P < 0.001 for CADC) from controls, except for duration of motility, which was not significant. Citrate inhibited baseline motility for 5 minutes before the introduction of both chemoattractants at time zero: control = 42.7%; citrate = 7.8%; control = 15.3%; citrate = 2.1%.

PMNL = polymorphonuclear leukocyte.

correlated with the release of LDH and cell death. The positive control samples (incubations of PMNL exposed to the respiratory burst stimulant alone) exhibited a 13.1% frequency of cell death, measured by the LDH assay. Cell death increased to 100% in the presence of both 12 mM citrate and the respiratory-burst stimulant.

DISCUSSION

Immediate treatment of the alkali-injured animal eye with SIMP has been reported to reduce the frequency of corneal ulceration by virtue of its antimetalloproteinase activity. Neutrophils are thought to be the predominant cell type that release substances destructive to tissues after alkali injury, and hence, are considered responsible for corneal ulceration. The paucity of PMNL in those corneas treated with SIMP, compared with the level found in control corneas, suggested that the mechanism of its ulcer protection in the alkali-injured eye might result, in part, from its exclusion of PMNL. In the current study, strong inhibition of chemoattractant of PMNL by SIMP in a collagenous substrate visual assay, confirms that its therapeutic effect is probably not solely caused by antimetalloproteinase activity. If used soon after the alkali injury, this powerful inhibitor of chemotaxis would be anticipated to inhibit invasion of the cornea by PMNL.

Prevention by SIMP of infiltration into the cornea would significantly reduce the amount of destructive proteases and oxygen radicals released into the corneal stroma, hence reducing the incidence of corneal ulceration after alkali injury.

Synthetic metalloproteinase inhibitor has previously been shown to stop the progression of established superficial ulcers in the alkali-injured cornea. Neutrophils, which had accumulated to create the corneal ulcer, largely disappeared by the end of the study. This earlier study stated that SIMP had no effect on chemotaxis, based on unpublished data using the under-agarose method. However, the results of the current study demonstrate that SIMP inhibits chemotaxis of PMNL and would have reduced the infiltration of additional PMNL, while inhibiting collagenase in and around the ulcer bed.

In the current study's findings, SIMP or citrate produced an early enhancement of the respiratory burst activated by an optimal concentration of the metabolic stimulant from alkali-degraded corneas, leading to PMN lysis and loss of activity in vitro. This response is similar to findings previously reported of PMN lysis in the presence of excessive amounts of the respiratory-burst stimulant. In spite of this finding SIMP and citrate appear to have only a positive effect on the clinical outcome in the alkali-injured eye.

Neutrophil activity, as part of the inflammatory process in the alkali-injured cornea, is a very complex
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Z

0.75 mM SIMP

0.1 mM SIMP

A 0.25 mM SIMP

> 0.50 mM SIMP

FIGURE 5. The effect of synthetic metalloproteinase inhibitor on the metabolic activity of polymorphonuclear leukocytes exposed to (A) opsonized zymosan or (B) the respiratory burst stimulant from alkali-degraded corneas. Each data point represents the mean ± SEM of five incubations of polymorphonuclear leukocytes. The positive control sample (•) consists of polymorphonuclear leukocytes stimulated with (A) 600 ng/ml opsonized zymosan or (B) a 1:50 dilution of the respiratory-burst stimulant. The negative control (•) consists of polymorphonuclear leukocytes alone.

process. Adherence of PMNL, chemotaxis, and respiratory-burst, with the release of proteases, oxygen radicals, and inflammatory mediators, form a sequence of events in the cornea leading to ulceration. Other types of cells can also contribute to tissue destruction, but only PMNL overwhelmingly dominate the cellular and inflammatory process in the corneal stroma. Although metalloproteinases are released from PMN and other sources, they are not the only destructive process leading to ulceration. It is important to avoid incriminating metalloproteinases alone as the cause of corneal ulceration, thereby ignoring the vast panoply of activities carried on by PMNL. Inhibition of metalloproteinases without reducing the numbers of PMNL present or accumulating, appears to be an inefficient way of preventing corneal ulceration. The current results suggest that reducing chemotaxis of PMNL with SIMP or citrate, prevents invasion of the cornea by PMNL. This is a direct and efficient means of decreasing corneal ulceration in the alkali-injured eye.

The use of ethanol as a diluent for the thiol peptide confounds the interpretation of prior animal experiments. Although ethanol was incorporated into the experimental and control solutions (2% to 5%) in both experiments, it is impossible to know if ethanol played any significant role in the outcome of any group. Our data—showing that ethanol suppresses PMN polarization, the first step in locomotion—and prior reports of inhibition of the respiratory burst suggest that ethanol contributed to the inhibition of PMNL in vivo. Despite this, SIMP clearly had a favorable effect on the prevention of corneal ulcers in both studies.

The effect of SIMP on resting PMNL is a compli-
cating feature of SIMP therapy. Synthetic metalloprotease inhibitor activates the respiratory burst of resting PMNL in a dose-dependent manner, with the lowest concentrations stimulating the most and the highest concentrations stimulating the least. The lowest concentrations of SIMP even enhance the opsonized zymosan-induced stimulation of PMNL, possibly as an additive effect. It is disquieting that a completely inactive PMN can be activated to undergo a dramatic respiratory burst on simple exposure to SIMP. In contrast, citrate does not have any effect on resting PMNL. In any case, that SIMP inhibits chemotaxis of PMNL is of greatest importance.

There are other significant differences between SIMP and citrate in their respective pharmacologic effects on PMNL. Synthetic metalloprotease inhibitor demonstrates a minimal effect on PMN polarization, but strong inhibition of chemotaxis in the CG-VCA system. This suggests that SIMP does not prevent the receptor–ligand interaction but does inhibit some step beyond that interaction in the chemotactic pathway. This is in contrast with citrate, which shows significant inhibition of both polarization and chemotaxis. The mechanism of action of citrate in its inhibition of PMNL relies on its calcium chelating properties. Both SIMP and citrate show an early enhancement of the respiratory burst in PMNL activated with the metabolic stimulant from alkali-degraded corneas, followed by cell lysis. Citrate exerts a powerful inhibitory effect on the adherence of PMNL to the vascular endothelium, potentially preventing a majority of PMNL from entering the cornea.

The effect of SIMP on adherence of PMNL is unknown but might well contribute greatly to its overall therapeutic effect. These findings add to our knowledge of the pharmacology of SIMP by showing its strong inhibition of chemotaxis of PMNL and its complex effect on the respiratory burst.

**Key Words**
alkali-degraded cornea, chemotaxis, citrate, polarization, polymophonuclear leukocytes, respiratory burst, synthetic metalloprotease inhibitor (SIMP)

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**References**


