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

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Purpose. To identify and synthesize the polymorphonuclear leukocyte chemoattractant(s) released from alkali-degraded corneas.

Methods. Corneas were degraded in 1.0 N NaOH, neutralized, ultrafiltered, and dialyzed. The final active ultrafilterate was subjected to high-performance liquid chromatography on a Protein PAK 160 column. The most active fractions were further separated on a μ-Bondapak-C₁₈ and 160 column in sequence.

Results. Fraction 38 from the final 160 column associated with a 210-nm absorption peak and elicited a polarization and chemotactic response from polymorphonuclear leukocytes. The loss of polarization activity in fraction 38 after exposure to prolidase suggests that this peptide contains a Pro-X (X = amino acid) peptide bond. The amino acid composition of fraction 38 was 35% glycine and 53% proline. Peptide sequence analysis was unable to establish a primary sequence even though Picotag analysis showed the presence of large amounts of the two amino acids. Mass spectrometry revealed only two molecular species of 312 MWt and 284 MWt. Tripeptides were synthesized using all possible amino acid permutations of 2 Pro and 1 Gly and tested in the polarization and chemotactic assays. These techniques demonstrated that n-acetyl-Pro-Gly-Pro, and to a lesser degree n-methyl-Pro-Gly-Pro, were the only synthetic tripeptides with activity similar to the purified chemoattractant.

Conclusions. The data show that the chemotactic peptides, purified from alkali-degraded whole cornea and confirmed with identical synthetic tripeptides, are N-acetyl-Pro-Gly-Pro and N-methyl-Pro-Gly-Pro. Although a number of proteins contain the Pro-Gly-Pro sequence, large amounts of collagen in the cornea suggest this as a major source. The small size and hydrophilic nature of these chemoattractants are predictive of a high degree of diffusibility. These chemoattractants are likely to play a major role in the early neutrophil response after an alkali injury.

Severe alkali injury to the eye results in vigorous infiltration of inflammatory cells into the cornea. The predominant cell type found is the polymorphonuclear leukocyte (PMNL), usually associated with the development of corneal ulcers. Polymorphonuclear leukocyte accumulation is generally accepted to result from the directional migration of cells in response to a chemoattractant gradient. The mediators that trigger the chemotactic response, therefore, are crucial to initiating the inflammatory response. Knowledge of the type of mediators released that stimulate the acute inflammatory response in the alkali-injured cornea is important to understanding the molecular mechanism of ulceration and to developing rational therapeutic regimens.

We have shown that mediators of PMNL activity are generated directly by alkali degradation of whole cornea. Our initial studies showed that alkali digestion of cornea in vitro generated a stimulant of the PMNL...
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respiratory burst and activated PMNL migration as measured by the Boyden chamber. Polymorphonuclear locomotion in the Boyden chamber studies was probably caused by the presence of a chemoattractant(s), but the technique was not sufficient to measure chemotaxis. Our more recent development of a collagen gel–visual chemotactic assay system permitted direct measurement of chemotaxis and cellular behavior in a simulated physiological environment. These studies revealed true PMNL chemotaxis in the low molecular weight ultrafiltrate.11

The purpose of the current study was to isolate, characterize, identify, and synthesize the low molecular weight PMNL chemoattractant(s) released from alkali-degradation of whole corneas. An additional aim was to determine if the purified and synthetic chemotactants elicit a chemotactic response from PMNLs.

MATERIALS AND METHODS

General

Hanks’ balanced salt solution (HBSS) was purchased from Gibco Laboratories (Chagrin Falls, OH). Calcium chloride, MgCl2, glutaraldehyde, sodium azide, Ficoll (type 400), and prolidase (proline dipeptidase, EC 3.4.13.9) were purchased from Sigma Chemical (St Louis, Mo). Sodium hydroxide, sodium phosphate monobasic, and sodium phosphate dibasic were obtained from Fisher Scientific (Fair Lawn, NJ). Sodium chloride was obtained from Mallinckrodt (Paris, KY). Hydrochloric acid was purchased from Ricca Chemical (Arlington, TX). Hypaque-76 was obtained from Winthorpe Laboratories (New York, NY). Leukotriene B4 (LTB4) was a generous gift from Merck Frosst Canada. Calcium chloride, MgCl2, glutaraldehyde, sodium azide, and sodium phosphate dibasic were obtained from Fisher Scientific (Fair Lawn, NJ). Sodium chloride was obtained from Mallinckrodt (Paris, KY). Hydrochloric acid was purchased from Ricca Chemical (Arlington, TX). Hypaque-76 was obtained from Winthorpe Laboratories (New York, NY). Leukotriene B4 (LTB4) was a generous gift from Merck Frosst Canada. (Point Claire–Dorval, Quebec, Canada).

Vitrogen 100 from Celtrix Laboratories (Palo Alto, CA) was used to prepare the collagen monomer solution for the collagen gel–visual chemotactic assay. The collagen solution consisted of 0.8 ml of Vitrogen 100 (2.5 mg collagen/ml; Celtrix), 0.1 ml of 0.1 N NaOH, and 0.1 ml of 10× phosphate-buffered saline (pH 7.3). This solution was mixed at 5°C and allowed to warm to room temperature immediately before use.

Polymorphonuclear Leukocyte 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. According to the technique of Ferrante and Thong,12 PMNLs were isolated from fresh heparinized human blood by centrifugation on Hypaque–Ficoll (density

\[ \text{Polarization Index} = 1.114 \] according to a previous article.13 Isolated PMNLs (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% PMNLs and ≈5% mononuclear cells and platelets, with the remaining percentage consisting of red blood cells. To measure the response of cells to chemoattractants, purified PMNLs were used in two separate systems, polarization and collagen gel–visual chemotactic assays. All incubation mixtures from both assays maintained an osmolality between 270 and 300, 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 assay14 was used as a preliminary screening test for large numbers of samples by quantitating the shape change that occurs after the exposure of PMNLs to a chemoattractant. Briefly, \( 1 \times 10^6 \) PMNLs were suspended in HBSS and phosphate-buffered saline in incubation mixtures to produce a physiological osmolality and pH for each sample or high-performance liquid chromatography (HPLC) fraction to be tested. Each incubation mixture (total volume = 250 μl) was then exposed to a sample in a stirred reaction chamber at 35°C for 5 minutes, unless otherwise noted. At the end of the incubation period, each cell suspension was mixed with an equal volume of 4% glutaraldehyde. Polymorphonuclear leukocytes in each sample were observed microscopically and assigned scores of 0 (resting = spherical with a smooth membrane), 1 (activated = irregular with uneven membranes), or 2 (polared = length ≡ width × 2).
was subtracted from each sample, and polarization activity was expressed as a percentage of the positive control. The collagen gel-visual chemotactic assay (CG-VCA) |l516 was used as the conclusive test for the presence of chemoattractant. The reference angle, derived from the displacement vector, equals 0° when the displacement is in a straight line toward the chemoattractant. Percent motility and stop frequency (percent of motile phase cell was stopped) were also calculated from the data.

To analyze directional movement further, the chemotactic indices was calculated at each minute. The formula for the chemotactic index of motile PMNLs incorporates the resultant vector length times the cosine of the reference angle divided by the total displacement (cos (θ)RVL/LD).

Each measurement was taken from zero to the time interval in question. The relative numbers, derived from this formula, are an index of the straightness of the PMNL path from +1.0 (direct movement toward chemoattractant) to −1.0 (direct movement away from attractant).

Alkali Degradation of Corneas
All experimental procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmalic and Vision Research. Bovine eyes were enucleated and frozen within 2 hours of death (Pel-Freeze Biologicals, Rogers, AR). These eyes remained frozen until the whole corneas (including epithelium and endothelium) were excised. In a preliminary experiment, three whole corneas were rinsed in HBSS for 30 seconds, their rims were blotted gently on paper towel, their wet weights were determined, and the corneas were dried. The average dry weight of each cornea was calculated to be 85 mg, and the average volume of water in each cornea was 500 μl.

A control experiment was performed to determine if the chemoattractant released from alkali-degraded corneas is endogenous to the uninjured cornea and capable of extraction by rinsing. Thawed corneas were treated with HBSS or alkali for 1 hour because exposure of thawed corneas in buffer for 24 hours at 37°C would pose a real risk of bacterial contamination, cellular disintegration, or both. Two separate groups of six corneas were pooled, rinsed, blotted, and treated with 6 ml of HBSS or 1 N NaOH (final concentration) at 37°C for 1 hour. This yielded

### TABLE 1. Polymorphonuclear Leukocyte Polarization Activity Generated by the Final HPLC Active Fraction 38 After Prolidase Treatment

<table>
<thead>
<tr>
<th>Sample</th>
<th>Incubation Time (hours)</th>
<th>Polarization Score</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Control</td>
<td>75 µl 100–3,000 UF + HBSS</td>
<td>2</td>
<td>85</td>
</tr>
<tr>
<td>Negative Control</td>
<td>75 µl HBSS + prolidase–HBSS</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Experimental</td>
<td>38 + prolidase–HBSS</td>
<td>2</td>
<td>34</td>
</tr>
<tr>
<td>Positive Control</td>
<td>100 µl fraction 38 + HBSS</td>
<td>4</td>
<td>63</td>
</tr>
<tr>
<td>Negative Control</td>
<td>100 µl HBSS + prolidase–HBSS</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Experimental</td>
<td>100 µl fraction 38 + prolidase–HBSS</td>
<td>4</td>
<td>15</td>
</tr>
</tbody>
</table>

Each sample contained equal amounts of chemoattractant, enzyme and HBSS where appropriate. The final volume of each PMNL incubation mixture was 500 μl. The negative control value was subtracted from each sample, and polarization activity was expressed as a percentage of the positive control (as in Table 1) or as specific polarization activity.

Collagen Gel–Visual Chemotactic Assay
The collagen gel–visual chemotactic assay (CG-VCA) |l516 was used as the conclusive test for the presence of chemoattractant activity. In this assay, the chemotactic movement of PMNLs through a collagen gel was measured directly by a computer-assisted visual tracking system. Immediately before the addition to the CG-VCA, the PMNL suspension was centrifuged at 60g for 4 minutes, and the collagen solution was simultaneously brought to room temperature. The PMNL pellet was gently resuspended in the collagen solution (2.0 x 10^7 PMNLs/ml), which was then added to the CG-VCA system and allowed to gel. Thick walled dialysis membrane (12,000 to 14,000 MWt cutoff pore size, Spectra/Per 4; [Spectrum Medical Industries, Houston, TX]) served as a semipermeable barrier between the collagen gel with PMNLs and the glass capillary tube containing the chemoattractant.

Polymorphonuclear leukocyte movement was characterized by analyzing the x and y coordinates of each cell at each time interval. The change in cell position from one time interval to the next was computed as follows: (Δx = xₙ - xₙ₋₁) and (Δy = yₙ - yₙ₋₁). Both values are in μm. Based on these calculations, the following cell behavioral characteristics were derived: displacement for a time interval (D = √(Δx² + Δy²)), resultant vector length (RVL = V/(D/minute), velocity (V = D/minute), and the angle of displacement with respect to the x-axis (θ = tan⁻¹[(Δy/Δx)]).

The scores of 100 PMNLs were added, producing a total polarization score. Negative control values (PMNLs in buffer only) were subtracted from all samples to achieve a polarization index (as in Fig. 1) or, in some cases, polarization activity was expressed as a percentage of the positive control (as in Table 1) or as specific polarization activity.

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a corneal dry weight to final volume of HBSS or alkali of 1:12. The samples were slowly titrated to pH 7.4 with HBSS or 2.0 N HCl, respectively, while stirring. After centrifugation of each neutralized sample (300g for 15 minutes), the supernatants were purified by ultrafiltration and dialysis as described in Ultrafiltration and Dialysis. The final samples, containing substances in the 100 to 1000 molecular weight range, were tested for activity in the polarization assay.

A separate experiment used a 24-hour exposure of corneas to alkali, optimizing the chemoattractant yield. Three whole corneas were pooled, rinsed, and blotted. The corneas were placed in 3 ml of 1.0 N NaOH (final volume and concentration of alkali includes the water content of each cornea) at 37°C for 24 hours. This yielded a corneal dry weight to final volume of 1.0 N NaOH of 1:12. The alkali-degraded corneas were slowly neutralized to pH 7.4 with 2.0 N HCl while stirring. The final volume of the completely dissolved corneas, after acid neutralization, was 4.5 ml.

The purification experiment of this crude suspension was performed three times with similar results. A description of the methods used in each case follows.

**Ultrafiltration and Dialysis**

The crude suspension (1.500 ml) was ultrafiltered through 30,000 MWt cutoff Centriprep (Amicon, Beverly, MA) concentrators at 1200g for 90 minutes. This active filtrate (1.250 ml) was then added to Spectra/Por cellulose ester dialysis membranes (Spectrum Medical) with a 100 molecular weight cutoff. The sample was dialyzed twice against 1 liter of distilled water containing 0.05% sodium azide for 1 hour each and once against 1 liter of HBSS for 1 hour. The active isonicotinic reagent (1.363 ml) was again ultrafiltered through 3000 MWt cutoff Centriprep (Amicon) concentrators at 2500g for 1 hour. This active filtrate (1.250 ml) was centrifuged through an Amicon MPS-1 micropartition system with a YM1 Diaflo membrane (1000 MWt). The final active filtrate (1 ml) was further purified by three successive HPLC columns.

**High-Performance Liquid Chromatography**

The sample (1 ml) was injected into the first Protein PAK 1-60 column (Waters Associates, Milford, MA), which separates on the basis of molecular size. The column was eluted by 0.05 M NaCl at 1.0 ml/minute, and the absorbance was monitored at 210 and 280 nm. The eluant was monitored at 210 and 280 nm and was collected into 0.5-ml fractions. The peak polarization response was observed in fraction 11. The balance of active fraction 11 (0.4 ml) was injected into a second 1-60 column and eluted by 0.05 M NaCl at 0.5 ml/minute. Fractions (0.25 ml) were monitored at 210 and 280 nm.

**Amino Acid Analysis**

An internal standard (alpha amino butyric acid) was added to an aliquot of the final active fraction. The fraction was dried using vacuum centrifugation and hydrolyzed (in vacuo) using gaseous phase 6 N HCl for 24 hours at 110°C. The sample was then dried again and derivitized using phenylisothiocyanate by the standard protocol recommended by the manufacturer for the PicoTag System (Waters Associates). The resultant phenylthiocarbamyl amino acids were analyzed using a Waters Associates PicoTag Column with buffers as recommended by the manufacturer. Standard amino acids (H), 6 N HCl, and phenylisothiocyanate were purchased from Pierce Chemical (Rockford, IL).

**Mass Spectrometry**

An aliquot of the final active fraction or synthetic peptides was subjected to matrix-assisted laser desorption ionization mass spectral analysis using two different matrices (4-nitrophenol and alpha-cyano-4-hydroxycinnamic acid).

**Peptide Sequence Analysis**

An aliquot of the final active fraction was applied to a polybrey treated glass fiber filter that had been precycled for four cycles. The sample was then put into the quartz cartridge and into a model 470A Applied Biosystems (Foster City, CA) protein sequencer with an online 120A PTH amino acid analyzer (Applied Biosystems). The methodologies used in the sequencer and the PTH analyzer were as recommended by the manufacturer. All chemicals and supplies used to generate the sequence analysis were purchased from Applied Biosystems.

**Treatment of Active Fraction With Prolidase**

Prolidase (116,000 MWt) in HBSS was added to the ultrafiltrate or an aliquot of the final active fraction at a concentration of 333 U/150 µl incubation mixture containing 500 µM Ca²⁺ and 600 µM Mg²⁺. All samples were incubated for 2 or 4 hours at 37°C and maintained at a pH of 8.0. One unit of prolidase will hydrolyze 1.0 µmole of Gly-Pro per minute under these conditions. At the end of the incubation period, each sample was passed through 100,000 MWt cutoff microspin ultrafilter (Lida Manufacturing, Kenosha, WI) to remove the prolidase and the pH of the filtrate returned to 7.4 before testing in the polarization assay.
Synthetic Peptides
Nine possible permutations of free, acetylated, or methylated N-terminal tripeptides with two prolines (P) and one glycine (G) were synthesized. The peptides (N-acetyl-PGP, N-acetyl-PPG, N-acetyl-GPP, PPG, GPP, N-methyl-PGP, N-methyl-PPG, and N-methyl-PPG) were manually synthesized on a Boc-Pro or Boc-Gly Merrifield resin (Millipore, Bedford, MA). Ninhydrin tests were used after each deblocking and coupling step to check for desired results. Amino acids were coupled using routinely available t-Boc synthetic techniques. N-methyl-amino acids were purchased from Sigma, but acetylated peptides were capped after removal of the t-Boc group.

The finished trimer was dried under vacuum and cleaved with anhydrous hydrogen fluoride. The cleaved product was washed with ether and extracted with 40% acetic acid. Both the ether wash and the acid wash were freeze dried. Mass spectral analysis indicated that the masses found in both washes agreed precisely with the expected masses of the target products.

Peptide samples were dissolved in 3% acetic acid and dialyzed (1 ml/l) in Spectra POR cellulose ester membranes with a 100-MWt cutoff (Spectrum) against distilled water with 0.05% sodium azide for 16 hours at 0°C to 4°C, and HBSS for 4 hours (changed every hour) at room temperature. The absorbance at 280 nm was magnified 20 times. The absorbance at 280 nm was measured 20 times. Acetonitrile was introduced in the eluant at fraction 40, and the absorbance was detected at 210 and 280 nm. The absorbance at 280 nm was magnified 20 times. Acetonitrile was introduced in the eluant at fraction 40, and the concentration increased to 100% by fraction 60. The peak polarization response was demonstrated in fraction 11 (0.5 ml).

RESULTS
Purification of the Chemoattractant(s) From Alkali-Degraded Corneas
The corneal samples treated with HBSS for 1 hour showed no significant activity (polarization index, mean ± SEM, n = 5: 10 µl = 1.2 ± 0.8, 25 µl = 1.8 ± 1.9, 50 µl = 1.3 ± 1.0, and 100 µl = 2.9 ± 2.4), whereas the corneas treated with alkali for 1 hour demonstrated a high degree of activity (polarization index, mean ± SEM, n = 5: 10 µl = 14.9 ± 5.1, 25 µl = 54.7 ± 7.2, 50 µl = 87.8 ± 8.0, and 100 µl = 98.3 ± 8.1).

The crude suspension from corneas degraded by alkali for 24 hours was ultrafiltered and dialyzed to produce an isotonic sample containing molecules in the 100- to 1000-MWt range. This low molecular weight ultrafiltrate retained 84% of the polarization activity found in the original ultrafiltrate (<30,000 MWt). Time–response curves for three doses of the low molecular weight ultrafiltrate demonstrated that the peak polarization response occurred at 3 to 5 minutes and decreased at 15 and 45 minutes (Fig. 1). Only 16% of the original polarization activity was detected in the retentates containing molecules in the 1000- to 30,000-MWt range. Retentates with >30,000 MWt were not analyzed for polarization or chemotactic activity because of the presence of a high molecular weight stimulant of the PMNL respiratory burst,17 which overrides the chemotactic response.

After injection of the 100- to 1000-MWt ultrafiltrate into the first I-60 HPLC column, the PMNL polarization assay detected peak activity in fraction 24 and moderate activity in fraction 25 (Fig. 2). The remainder of fraction 24 (0.4 ml) was injected into a µ-Bondapak-C18 column. Activity was demonstrated by the polarization assay in fractions 10 to 12, with the peak at fraction 11 (Fig. 3). The balance of fraction...
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TABLE 2. Amino Acid Analysis of Fraction 38

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proline</td>
<td>52.5</td>
</tr>
<tr>
<td>Glycine</td>
<td>35.1</td>
</tr>
<tr>
<td>Alanine</td>
<td>5.1</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>3.9</td>
</tr>
<tr>
<td>Serine</td>
<td>1.6</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.1</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.0</td>
</tr>
</tbody>
</table>

FIGURE 4. Chromatography of the polymorphonuclear leukocyte chemoattractant from alkali-degraded corneas on Protein PAK I-60 high-performance liquid chromatography column. The balance of active fraction 11 (0.4 ml) from the C₄₀ column was injected into a second I-60 column and eluted by 0.05 M NaCl. Fractions (0.25 ml) were collected at 0.5 ml/minute. The absorbance of each fraction was monitored at 210 and 280 nm. The polarization assay detected an equal amount of activity in fractions 38 and 39 (0.25 ml each).

11 (0.4 ml) was rechromatographed on the Protein PAK I-60 column. The chromatogram demonstrated one major 210-nm absorption peak occurring in fractions 38 and 39, and one minuscule 280-nm peak appearing in fraction 39 (Fig. 4). The polarization assay detected an equal amount of activity in fractions 38 and 39. Fraction 38 is likely to be more pure because it has little absorbance at 280 nm. The molecular weight of active fraction 38 is located between the standards tyrosine (181) and reduced glutathione (307), suggesting a size in this general range (Fig. 5). Comparison of the specific activities calculated for the crude ultrafiltrate (<30,000 MWt cutoff, and the purified active fraction (#38) showed a 6875-fold purification of the chemoattractant. The remainder of fraction 38 was used to analyze the molecular characteristics and chemotactic activity of the chemoattractant.

The ultrafiltrate (100 to 3000 MWt cutoff) or active fraction 38 was mixed with prolidase (E.C. 3.4.13.9) and incubated for 2 or 4 hours. After the 4-hour incubation, PMNL polarization activity was decreased by 88% for the ultrafiltrate sample and 77% for fraction 38 (Table 1). This highly specific enzymatic reaction, cleaving Pro-X (X = amino acid) peptide bonds, is strong evidence that the chemoattractant is a peptide containing the Pro-X peptide bond.

Analysis of fraction 38 shows approximately twice as much proline as glycine based on amino acid composition by the Picotag method. There are no other amino acids present in high enough percentages to be part of this small peptide (Table 2). The above information strongly suggested that the chemoattractant is a tripeptide with two prolines and one glycine.

N-terminal sequence analysis was unable to establish a primary sequence, but Picotag analysis showed an abundance of sample. This indicates blockage of the N-terminal amino acid residue.

FIGURE 5. Chromatography of standards on Protein PAK I-60 high-performance liquid chromatography column. The column was eluted by 0.05 M NaCl and 0.25 ml fractions were collected at 0.5 ml/minute. The absorbance was monitored at 210 nm. The location of the molecular weight standards, tyrosine (181) and reduced glutathione (307), suggest that active fraction 38 is within this molecular weight range. GSH = reduced glutathione; GSSG = oxidized glutathione.

FIGURE 6. Matrix-assisted laser desorption ionization mass spectral analysis of active fraction 38, using a 4-nitrophenol matrix. The two unknown molecules are at molecular weights 312 and 284.
TABLE 3. Specific Activity of Synthetic Peptides in Polymorphonuclear Leukocyte Polarization Assay

<table>
<thead>
<tr>
<th>Synthetic Peptide</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-acetyl-PGP</td>
<td>17.0</td>
</tr>
<tr>
<td>n-methyl-PGP</td>
<td>9.7</td>
</tr>
<tr>
<td>PGP</td>
<td>4.4</td>
</tr>
<tr>
<td>PPG</td>
<td>2.7</td>
</tr>
<tr>
<td>n-acetyl-GPP</td>
<td>1.7</td>
</tr>
<tr>
<td>GPP</td>
<td>0.7</td>
</tr>
<tr>
<td>n-methyl-GPP</td>
<td>0.2</td>
</tr>
<tr>
<td>n-methyl-PPG</td>
<td>0.2</td>
</tr>
<tr>
<td>n-acetyl-PPG</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Active fraction 38</td>
<td>21.0</td>
</tr>
</tbody>
</table>

One unit activity was defined as the amount of sample required to generate a 50% polarization index. Specific polarization activity was calculated as units of activity per milligram peptide.

Matrix-assisted laser desorption ionization mass spectrometry showed that fraction 38 contains only two unknown molecules of 312 MWt and 284 MWt (Fig. 6). Two different, but mutually confirmatory, matrices were used.

Synthetic Peptides

The polarization response of PMNLs was used to screen synthetic peptide samples before selected chemoattractant studies in the CG–VCA system. In summary, Table 3 shows that N-acetyl-PGP and N-methyl-PGP produced a powerful response in the polarization assay approximately an order of magnitude greater than the less active peptides. The specific activity of N-acetyl-PGP was similar to the purified chemoattractant in fraction 38. One unit activity was defined as the amount of sample required to generate a 50% polarization index. Specific polarization activity was calculated as units of activity per milligram of peptide. Note that the molecular weights of n-acetyl-PGP and n-methyl-PGP are 312 and 284 respectively, the same as that noted by mass spectral analysis of the purified chemoattractant.

Time–response curves for three doses of N-acetyl-PGP and N-methyl-PGP demonstrated that the peak polarization response was at 3 to 5 minutes for each dose of both tripeptides, decreasing at 15 and 45 minutes (Figs. 7, 8).

Chemotactic Activity of Fraction 38 and Synthetic Peptides

Polymorphonuclear leukocyte chemotaxis was demonstrated in the CG–VCA system using the purified active fraction (38) from alkali-degraded corneas (Fig. 9). The chemotactic index reached a peak at 2 minutes, decreasing for the remaining 8 minutes of the experiment.

N-acetyl-PGP demonstrated PMNL chemotaxis in the CG–VCA system (Fig. 10), comparable to the response found with fraction 38. A significantly positive chemotaxis was noted from 7 to 10 minutes. There was a delay in the chemotactic response, probably repr...
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FIGURE 9. Chemotactic index of polymorphonuclear leukocytes exposed to purified active fraction 38 from alkali-degraded corneas. Active fraction (1.8 × 10⁻⁴ M, total estimated concentration of N-methyl-PGP and N-acetyl-PGP from Picotag analysis of amino acid composition) was added to the glass capillary tube. Each data point represents the chemotactic indices (mean ± SEM) for both 30-second intervals at each minute for all motile polymorphonuclear leukocytes from three separate experiments. The number of motile cells is included adjacent to the data point for each time period. Ninety-nine cells were tracked. The single sample Student’s t-test was used to determine if the chemotactic index for each minute differed significantly from zero (greater than zero from 2 to 10 minutes, \( P < 0.001 \)). Polymorphonuclear leukocytes also were tracked for 5 minutes before the addition of the chemoattractant as a control, but the number of motile cells was low, and their movement was not significantly different from zero (random).

A similar chemotactic response was recorded for PMNLs exposed to synthetic N-acetyl-PGP in the CG-VCA system (Fig. 11). A moderate chemotaxis was reached at 3 minutes and maintained thereafter.

The behavioral characteristics of PMNLs exposed to the active fraction 38, N-acetyl-PGP, and N-methyl-PGP are summarized in Table 4. There was a dramatic increase in the percent motility of each PMNL population after the addition of each chemoattractant. The stop frequency of PMNLs during the 5- to 10-minute period after the addition of each chemoattractant showed a significant decrease \( (P < 0.001, \) Student’s t-test) when compared to the 5-minute control period. Based on the Student’s t-test, the velocity of PMNL movement during the 5- to 10-minute period was significantly increased over the 5-minute control period for N-acetyl-PGP \( (0.02 < P < 0.05) \), N-methyl-PGP \( (P < 0.001) \), and active fraction 38 \( (P < 0.001) \). When compared to the respective 5-minute control periods, PMNL displacement was not significantly increased during the 5- to 10-minute period for N-acetyl-PGP, but it was highly significant \( (P < 0.001) \) for N-methyl-PGP and the active fraction.

FIGURE 10. Chemotactic index of polymorphonuclear leukocytes exposed to synthetic N-methyl-PGP. N-acetyl-PGP (2.8 × 10⁻³ M) was added to the glass capillary tube. Each data point represents the chemotactic indices (mean ± SEM) for both 30-second intervals at each minute for all motile polymorphonuclear leukocytes from three separate experiments. This number of motile cells is included adjacent to the data point for each time period. One hundred three cells were tracked. The single sample Student’s t-test was used to determine if the chemotactic index for each minute differed significantly from zero (greater than zero at 7 \( [0.01 < P < 0.02] \), 8, 9, and 10 minutes \( [P < 0.001] \)). Polymorphonuclear leukocytes also were tracked for 5 minutes before the addition of the chemoattractant as a control, but the number of motile cells was low, and their movement was not significantly different from zero (random).

FIGURE 11. Chemotactic index of polymorphonuclear leukocytes exposed to synthetic N-methyl-PGP. N-methyl-PGP (1.5 × 10⁻³ M) was added to the glass capillary tube. Each data point represents the chemotactic indices (mean ± SEM) for both 30-second intervals at each minute for all motile polymorphonuclear leukocytes from three separate experiments. This number of motile cells is included adjacent to the data point for each time period. One hundred five cells were tracked. The single sample Student’s t-test was used to determine if the chemotactic index for each minute differed significantly from zero (greater than zero from 3 to 10 minutes \( [P < 0.001] \)). Polymorphonuclear leukocytes also were tracked for 5 minutes before the addition of the chemoattractant as a control, but the number of motile cells was low, and their movement was not significantly different from zero (random).
DISCUSSION

Our previous studies\textsuperscript{9,10,11} demonstrated the presence of PMNL chemoattractant(s) generated from alkali-degraded whole corneas. Its small size and hydrophilic nature imparted ideal characteristics for the rapid diffusion of the molecule through collagenous tissues.\textsuperscript{11} We conclude from the current study that the PMNL chemoattractants, isolated from these alkali-degraded corneas, are acetylated and methylated tripeptides (Pro-Gly-Pro). The absence of activity in corneas rinsed with HBSS demonstrated that the chemoattractants were not endogenous to the uninjured cornea.

The chemoattractant(s) released from alkali-degraded corneas, as well as the synthetic N-acetyl-Pro-Gly-Pro and the N-methyl-Pro-Gly-Pro, induced a peak polarization response by PMNLs within 5 minutes that decreased dramatically after 15 and 45 minutes. The basis for declining polarization activity at the latter time periods is unknown. It might indicate that the cell membrane receptors of the PMNL were saturated with the chemoattractant or that the cell was metabolizing the chemoattractant.

Alkali degrades the cornea into numerous heterogeneous peptides separable by column chromatography. Passage of the alkali-degraded corneal ultrafiltrate through a protein PAK 1-60 column and then the \(\mu\)-Bondapak-C\(_{18}\) column removed a large amount of the inactive peptides. A highly purified fraction (38) with chemotactic activity for PMNLs was recovered upon rechromatography on the protein PAK 1-60 column.

The PMNL chemoattractant(s) was determined to be a peptide with at least one Pro-X peptide bond, based on the loss of biologic activity after incubation with prolidase. The amino acid composition studies showed that the sample contained twice the amount of proline than glycine. Mass spectrometry revealed that only two unknown molecular species were present, with masses at 312 and 284, respectively. This information strongly suggested that the chemoattractant was a tripeptide with two prolines and one glycine. There were no other amino acids present in high enough percentages to be part of this peptide. An attempt at N-terminal sequencing failed, establishing that both tripeptides are probably N-terminal blocked. Calculation of the molecular weight of two prolines and one glycine, and acetylation or methylation of the N-terminal group, agrees exactly with the molecular weights of 312 and 284 found by mass spectrometry.

The limited permutations of free N-terminal, N-acetylated, or N-methylated tripeptides, containing two prolines and one glycine, made it practical to synthesize all nine possible sequences. Polarization assay of PMNLs exposed to these samples showed that only N-acetyl-PGP and, to a lesser degree, N-methyl-PGP yielded a potency comparable to the purified chemoattractant. Further analysis of both synthetic tripeptides in the CG–VCA system revealed chemotactic activity comparable to that of the purified chemoattractant. These results confirm that N-acetyl-PGP and N-methyl-PGP are PMNL chemoattractants. These data show that the alkali-generated chemoattractant and the synthetic chemoattractants are the same.

Acetylation\textsuperscript{18,19} is the most common form of naturally occurring N-terminal peptide blockage, but methylation\textsuperscript{20} also occurs. Any of these acetylated or methylated proteins with the sequence Pro-Gly-Pro from the amino terminus could be a source of the
chemoattractant. Alternately, exposure to alkali is known to cleave the ester-linked acetyl group from naturally occurring neutral peptides, potentially making these groups available to alkali-generated peptides with a free N-terminus. Alkali has been shown to cleave protein (including collagen) into heterogeneous peptide units. The amino terminus of these residual peptides would be susceptible to acetylation or methylation under these alkaline conditions. In fact, peptides are routinely acetylated at the N-terminus under alkaline conditions during peptide synthesis. The results of experiments in the current study show that this process could occur in the alkali-degraded cornea. Therefore, a parallel can be drawn for this process in the alkali-injured cornea.

Identification of the primary sequence of the purified chemoattractant opens an important door to basic studies and understanding of PMNL influx into the alkali-injured cornea. This knowledge will allow the synthesis of relatively large amounts of the chemoattractant for extensive testing. It also may allow for a rational plan of attack to defeat the destructive PMNL infiltration into the alkali-injured cornea by developing inhibitors of the chemoattractant. In this regard, the synthetic N-acetyl-Pro-Pro-Gly has no apparent chemotactic activity and, hence, is a candidate as a chemoattractant inhibitor. The other synthetic tripeptides with specific chemotactic activity <1.0, N-methyl-PPG, N-methyl-GPP, and unblocked GPP, might also be potential inhibitors. The precedent for this approach was set in an experiment in which PMNL accumulation, induced by injection of N-formyl-methionyl-leucyl-phenylalanine into normal cornea, was inhibited by the simultaneous injection of inactive, synthetically produced analogues. The search for inhibitors might be focused by using structure-based drug design, a recent innovative approach for developing new drugs as therapeutic agents.

The tripeptide P-G-P probably originates from a protein that is degraded by alkali. The National Center for Biotechnology Information Peptide Sequence Data Base was consulted to determine the number of proteins that contain the P-G-P sequence and its frequency in particular proteins. Numerous mammalian proteins contain this sequence, including collagen, proteoglycans, fibronectin, laminin, ICAM-1, integrin, and Na"+-K"+ ATPase. Collagen is one of the likeliest sources because of the relative frequency of this amino acid sequence in collagen and the fact that a relatively high proportion of corneal protein is collagen. The triple helical portion of the collagen molecule contains glycine in every third residue, with proline and hydroxyproline appearing next most frequently in the sequence. When the amino acid residues are cleaved, hydrolysis of the peptide bond renders a free carboxyl terminal while simultaneously, or shortly thereafter, methylation or acetylation of the N-terminal group might occur. The Pro-Gly-Pro sequence does exist in this portion of the collagen molecule.

Our current findings that N-acetyl-PPG and N-methyl-PPG induce chemotaxis in resting PMNL are the direct result of experiments designed to identify the chemoattractant activity in alkali-degraded whole corneas. Earlier studies showed a locomotory response by fibroblasts to collagen-derived peptides, especially tripeptide sequences selected for study based on their common appearance in the collagen molecule. Using similar reasoning, Laskin et al synthesized tripeptides, including combinations of Pro, Hyp and Gly, which activated PMNL locomotion in selected sequences. Polyytripptides, composed of 5 or 10 recurring units of Pro-Pro-Gly and Pro-Hyp-Gly, were the most active. Blocking of the amino or carboxyl end of the molecules changed the activity. No PGP sequence was tested. It is now known that the methodology used in the former experiment could only show locomotion of fibroblasts, whereas the latter experiment used checkerboard analysis of the Boyden chamber assay, a questionable method for simulating PMNL chemotaxis in vivo. The true chemotactic properties of these compounds, as measured by more recent and accurate visual techniques, might be regarded as unknown. In spite of these considerations, this family of compounds might indeed induce chemotaxis by attaching to the same PMNL membrane receptor as N-acetyl-PPG and N-methyl-PPG.

It is likely that the alkali-generated tripeptide chemoattractant(s) discussed in this article play a significant role in triggering the early neutrophil response after an alkali injury. Other low molecular weight chemoattractants, such as platelet-activating factor and LTB4, are known to be present in the experimental alkali-injured eye and may be active in the further recruitment and accumulation of neutrophils. It is yet to be determined whether other corneal inflammatory diseases generate the same tripeptide chemoattractant(s) as those discovered in the current study. These tripeptide chemoattractant(s) might be the common denominator in inflammatory conditions when degradation of collagen or other PGP-containing proteins is involved. Discovery of the alkali-generated chemoattractant(s) might lead to a fuller understanding of chemotaxis in alkali-injuries as well as in other eye diseases and inflammation in other tissues of the body.

Key Words
chemoattractant, chemotactic peptides, synthetic peptides, polymorphonuclear leukocytes, alkali injury, cornea, ulceration
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