Alkali-Degraded Cornea Generates a Low Molecular Weight Chemoattractant for Polymorphonuclear Leukocytes

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Purpose. The current study was designed to determine if a polymorphonuclear leukocyte (PMN) chemoattractant is derived from alkali-degraded whole cornea and to establish a range for its molecular weight.

Methods. We utilized a collagen gel-visual chemotactic assay to quantify the directional movement of PMN exposed to alkali-degraded corneas (30 min or 24 hr). In this experiment, the sample to be tested for chemotactic activity passed through a 14,000 molecular weight cutoff membrane into a collagen gel in which resting neutrophils were suspended in a random fashion. Cell movement was videotaped and subsequently tracked by digitizing the centroid of the cells at 30-sec intervals. Computer analysis of these tracks illustrated many behavioral characteristics, including directional movement.

Results. Alkali-degraded whole bovine corneas produced a chemotactic response in neutrophils within 5 min. Dilution of the 24-hr sample to 1:7 yielded a significant chemotactic response. The chemotactic response of both the 30-min and 24-hr samples followed a dose-response curve.

Conclusions. This agent may be one of the inflammatory mediators that trigger the early neutrophil response after an alkali-injury to the eye. Invest Ophthalmol Vis Sci. 1993;34:2297-2304.
MATERIALS AND METHODS

Materials

Hanks balanced salt solution (HBSS) was purchased from Gibco (Chagrin Falls, OH). Calcium chloride, MgCl₂, and Ficoll were purchased from Sigma (St. Louis, MO). Hypeaque-M 90% was obtained from Winthrop (New York, NY). Vitrogen 100 (Celite, Palo Alto, CA) was used to prepare the collagen monomer solution. Thick-walled dialysis membrane (Spectrum Por 4, 12,000–14,000 molecular weight cutoff, Spectrum Medical, Los Angeles, CA) was used to cover the tips of the capillary tubes.

Preparation of Solutions

All HBSS solutions contained Ca²⁺ (500 µmol/l) and Mg²⁺ (600 µmol/l). The collagen solution consisted of 0.8 ml of Vitrogen 100 (2.5 mg collagen/ml), 0.1 ml of 0.1 N NaOH, and 0.1 ml of 10× phosphate-buffered saline (pH 7.3) at 5°C.

Alkali Treatment of Corneas

Frozen bovine eyes were purchased from Pel-Freez (Rogers, AR). Pel-Freez enucleates and freezes eyes within 2 hr of the death of the animal. In our laboratory, whole corneas were excised from the frozen bovine eyes. A preliminary experiment determined the dry-weight to wet-weight ratio of the average cornea. The test samples were established by calculating the amount of water in each batch of ten pooled corneas and adding the amount of alkali to achieve a final concentration of 1 N NaOH. The ratio of tissue dry weight to alkali volume was 1:12 (wt/vol). In these experiments, ten corneas represented a dry weight of 1 g and were immersed in a total of 12 ml of 1 N NaOH. The samples were incubated at 37°C for 30 min or 24 hr. The extraneous fluid covering the corneas was recovered from the 30-min sample. Corneas from the 24-hr sample were completely dissolved in the NaOH. The respective suspensions were subsequently titrated, while stirring, to pH 7.4 with 4 N HCl and centrifuged at 15,000 × g for 15 min. The supernatants were placed in 100 MWCO dialysis tubing (Spectrum Medical) and dialyzed twice in distilled water (1 ml/l) for 2 hr to remove salts. The samples were then made isotonic by adding 10X HBSS to the retentate (0.5:10.0, vol/vol). They were subsequently diluted and tested for their capacity to activate PMN chemotaxis.

Neutrophil Isolation

This study was approved by the institutional review board of Brookwood Medical Center, and the tenets of the Declaration of Helsinki were followed. All blood donors signed consent forms explaining the nature and possible consequences of venipuncture. Blood was collected from only one donor each day for a total of 7 days, forging a natural randomized block design. Using a published technique, the PMN were isolated from fresh heparinized human blood by centrifugation on Hypaque–Ficoll (density, 1.14), according to a previous article. The purity of this cell suspension was 89% ± 5.6% PMN (mean ± standard error of the mean, n = 7) with 6% red blood cells, and the remaining 5% consisted of platelets, lymphocytes, and eosinophils. The viability of the PMN population was 96–99%. Isolated PMN were resuspended in HBSS at room temperature and gently inverted a single time at 10-min intervals. Immediately before addition to the chemotactic chamber, the cell suspension was centrifuged at 60 × g for 4 min while the collagen solution was brought to room temperature. The cell pellet was gently resuspended in the collagen solution (2.0 × 10⁷ PMN/ml). This concentration of PMN allowed for sufficient dispersal of these cells in the gel matrix; therefore, the risk of cell collisions during the 12-min tracking period was virtually excluded.

Collagen Gel-Visual Chemotactic Assay (CG-VCA)

Equipment. In a previous article, we presented a detailed description of the method of the CG-VCA system for measuring PMN chemotaxis. The description of the CG-VCA assay in the current publication has been edited to reflect the modifications that were made in the technique. Two glass capillary tubes (1.54 mm outer diameter and 1.08 mm inner diameter), their ends covered by one layer of dialysis membrane (14,000 MWCO), were inserted into opposite ends of a short piece of polyethylene tubing (1.6 mm inner diameter). The compartment formed between the opposing membranes was 0.5 mm long and 1.6 mm in diameter. The compartment formed between the opposing membranes was 0.5 mm long and 1.6 mm in diameter. A 0.5-mm square opening was made in the top and bottom of the compartment, directly opposite each other, leaving a thin strip of the polyethylene sleeve on each side.

The apparatus was positioned on the stage of a Zeiss standard research microscope (Norcross, GA) on the notched rim of a plastic immersion chamber (height, 10 mm; inside diameter, 10 mm) containing 400 µl of HBSS, bringing the level to just below the compartment (Fig. 1). The chemotactic compartment was filled with 3 µl of the solution of monomeric collagen containing the suspension of PMN. A glass cover slip was sealed to the top of the immersion chamber with a mixture of paraffin wax and white petrolatum (3:1), creating a humidified chamber. The lower surface of the glass cover slip was in contact with the collagen suspension. This suspension was then incubated for 30 min at 36.5 ± 0.5°C, resulting in the for-
FIGURE 1. Detailed diagram of sample chamber. Exploded views show two glass capillary tubes, tipped with a dialysis membrane (14,000 MWCO), snugly fitted into either end of a polyethylene tubing sleeve to create a compartment 0.5 mm long and 1.6 mm in diameter. Openings in the upper and lower portion of the compartment allow unrestricted microscopic observation. The compartment is filled with 3 μl of a monomeric collagen solution, containing suspended PMN. A three-dimensional hydrated gel of native collagen fibrils forms within 30 min. The top view shows an unrestricted light path. The side view shows a glass cover slip in contact with the collagen matrix. (Reprinted with permission and minor modifications from The Journal of Immunological Methods (1991; 141:41–52), copyright 1991.

formation of a three-dimensional hydrated gel of native collagen fibrils. A heated water jacket and infrared incubator (Opti-Quip, Highland Mills, NY), with a temperature probe inside the immersion chamber, maintained a constant temperature. The solidified collagen gel was then immersed in prewarmed HBSS, using a portal at the bottom of the immersion chamber, allowing for the ionic constituents to diffuse into the collagen gel. After 15 min, the medium was withdrawn and again replaced with 400 μl of fresh prewarmed HBSS to just below the collagen gel compartment to prevent evaporation from the gel. To initiate development of the chemotactic gradient at time 0, 90 μl of the test sample was introduced into one glass capillary tube while HBSS (negative control) filled the opposite capillary tube. The test sample was alternated between the right and left capillary tube for each sample. A gradient formed in the collagen gel within a period determined by the diffusion coefficient and the concentration of the chemoattractant. The pH and osmolality range for all solutions was 7.3–7.4 and 280–300 mOsm, respectively.

The apparatus was oriented on the stage so that transillumination occurred through the openings in the compartment containing the gel. A 10X ocular with 16X planachromat objective lens was used to focus in the middle of the collagen gel (using a micrometer scale on the focusing adjustment) halfway between the dialysis membranes.

Cell Tracking. PMN movement and behavior, within the focal plane, were recorded for 5 min before and 12 min after the addition of the test sample using a Sanyo CCD video camera (Compton, CA) (without a lens) connected to a Panasonic AG 6200 video cassette recorder (Secaucus, NJ) and a color video monitor. A phototube linked the microscope and camera (length, 6.5 cm) so that the same focal plane in the collagen gel was viewed by the microscope oculars or the video monitor at 16X. The occasional red blood cell was omitted by identification. The camera was oriented so the test sample entered the video monitor image from either the right or left side. A central rectangular area, equivalent to 70% of the screen, was marked on the video monitor corresponding to a 392 X 294-μm area.
in the central portion of the collagen compartment. Only those cells located in the box distinctly visible as a digitized image were entered into the study. Using the video display, PMN were tracked by a double-blinded observer for 12 min using an International Business Machines AT-compatible computer from JC Information Systems (JC LIPS 286 base unit, 20/8 MHz, Fremont, CA) interfaced with a Logitech (Fremont, CA) Trackman mouse. Only 5 of 379 motile PMN left the tracking area during the experiment. The data from these cells was included up to their point of departure. Video analysis software from Jandel Scientific (JAVA version 1.2, Corte Madera, CA), interfaced with a TARGA M8 digitizer (Truevision, Indianapolis, IN), was used to capture images and store (American Standard Code for Information Exchange file to hard or floppy disc) the x and y coordinates of PMN locations. The JAVA software program was set so that every pixel in the digitized image was accessible by using the following override commands: fgar-1, mrange = 4, and targaset = M8A50050b. This program was also used to calibrate the system so that one pixel in the captured video image corresponded to 0.926 μm in the microscopic field. The track coordinates from each cell were saved at 30-sec intervals from 0–12 min. Only movements greater than 1.9 μm, during successive time intervals, were recorded. PMN moving less than 20 μm of total displacement and 8 μm of resultant vector length during the 12-min observation period were considered nonmotile cells.

**Image Analysis.** The ASCII files of the x and y coordinates collected by JAVA were loaded into Lotus 1-2-3, release 3.0 (Lotus Development Corporation, Cambridge, MA). A spreadsheet requiring extended memory was prepared to analyze the data, transforming it through formulas to calculate various parameters. The change in cell position from one time interval to the next was computed as follows:

\[(\Delta x = x_n - x_{n-1})\]

and

\[(\Delta y = y_n - y_{n-1}),\]

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

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

resultant vector length

\[(RVL = \sqrt{(x_n - x_i)^2 + (y_n - y_i)^2}),\]

velocity \((V = D/min)\), 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 and 180° when the displacement is in a straight line directly away from the chemoattractant. Further measurements were calculated from the data, such as the percent motility, stop frequency (percent of motile phase cell was stopped), and duration of motility (number of minutes from the first to the last movement).

To analyze directional movement further, the chemotactic indexes was calculated at each minute. The formula for the chemotactic index of motile PMN incorporates the resultant vector length times the cosine of the reference angle divided by the total displacement

\[
\frac{\cos(\text{Ref } \theta) \times RVL}{\sum D}.
\]

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 PMN path from +1.0 (direct movement toward chemoattractant) to −1.0 (direct movement away from attractant).

**Statistics**

Experiments were performed to determine the dose response for activation of PMN chemotaxis by alkali-treated corneas. Following a randomized balanced incomplete block design, the various behavioral characteristics for experimental samples were evaluated for specific differences from the control by the Dunnett’s test for analysis of variance. Each population of cells was defined as the number of tracked cells in three separate collagen gels exposed to the same sample.

**RESULTS**

Twenty-four-hour alkali-degraded whole bovine cornea generated an inflammatory mediator that was chemotactic for PMN. Dose–response curves showed a progressively greater chemotactic response from a dilution of 1:21 up to 1:7, the latter was statistically significant \((P < 0.05, \text{Fig. 2A})\). The undiluted solution showed an earlier rise in chemotaxis but an overall
FIGURE 2. Chemotactic indexes of PMN responding to alkali-degraded bovine cornea. Data points represent the mean ± the standard error of the mean of the chemotactic index for all motile PMN at each minute. (A) Curves represent dilutions of the dialyzed supernatant resulting from 24-hr alkali-degradation of corneas. The number of motile PMN of the total number tracked for each sample was as follows: 1:21 dilution, 61 of 93; 1:14 dilution, 69 of 90; 1:7 dilution, 69 of 97; and undiluted, 62 of 87. (B) Curves represent the dilutions of the dialyzed supernatant resulting from 30-min alkali-degradation of corneas and the HBSS control sample. The number of motile PMN of the total number tracked for each sample was as follows: 1:21 dilution, 37 of 93, 1:7 dilution, 61 of 92; and HBSS control, 26 of 83. The early time intervals (1 and 2 min) of the HBSS control and 1:21 dilution curves were composed of few n values, giving the erroneous impression that positive or negative chemotaxis had occurred.

slight fall off from the 1:7 dilution. The chemotactic response using a 30-min interval of alkali treatment followed a similar pattern as noted with the 24-hr sample (Fig. 2B). In these experiments, the control samples showed random locomotion.

Each population was examined with respect to a specific set of behavioral patterns (Table 1). For example, the percent of motile PMN at all the dilutions increased significantly to 67-72% from the control level of 18% after exposure to the 24-hr alkali-injured cornea. The 30-min alkali-treated cornea showed a lesser increase to 39% for the 1:21 dilution but a significant increase to 63% for the 1:7 dilution.

When analyzing only motile cells, the stop frequency decreased significantly from the control level of 39.3% to 12.3-17.5% with exposure to all dilutions of the 24-hr corneal sample. This decrease was also significant in the 1:7 dilution of the 30-min sample, with a drop to 21.3%.

The velocity of PMN increased significantly from the control rate of 5.2 μm/min to a range of 9.3-9.9 μm/min using the 1:14, 1:7, and undiluted 24-hr samples. The velocity noted with the 1:21 dilution was elevated to 8.8 μm/min, but this was not statistically significant. The 1:7 dilution of the 30-min sample was increased but not statistically significant.

The total displacement of PMN followed a dose-response curve, which was significant at every dilution of the 24-hr sample, compared with the control. The displacement of the 1:7 dilution of the 30-min sample was increased but not statistically significant.

A dose-response curve reflecting the resultant vector length showed elevation at most points, but only the 1:7 dilution of the 24-hr sample was significantly different.

The duration of motility of PMN exposed to any of the alkali-treated samples did not differ from that of the controls.

DISCUSSION

The current study presents the only known low molecular weight PMN chemoattractant generated directly from any tissue by alkali degradation. This extends our prior studies with modified Boyden chambers, which provided evidence that a PMN locomotion-inducing agent was produced from alkali-degraded collagen preparations or corneal tissue. In the current experiments, the chemoattractant rapidly passed through 14,000 MWCO pores in dialysis membranes and across a collagen gel matrix. This demonstrates that the chemotactic factor has a relatively low molecular weight and is readily diffusible in a collagenous tissue. We believe that this alkali-induced chemoattractant might play a major role in triggering the early neutrophil response after an alkali-injury to the eye.

This newly discovered chemotactic factor and the powerful, bench-mark chemoattractant leukotriene B₄ cause PMN to respond in a similar way with respect to
their response times, motility patterns, and chemotactic indexes. The only differences are the narrower range of concentrations over which the alkali-generated chemoattractant was chemotactic and a stronger chemokinetics at its lowest doses.

The CG-VCA system described in this article simulates PMN movement in the cornea by using a collagen matrix. This matrix is different from the cornea in that there is more water within these hydrated collagen gels and the fibrils are randomly oriented. Although there are other differences, including the absence of mucopolysaccharides, the similarity to cornea is much greater than that noted in any other system. Furthermore, except for denaturation of the collagen, the changes that occur after alkali injury of the cornea might make our in vitro system more similar to the clinical situation (ie, loss of glycosaminoglycans, increased hydration, and partial loss of collagen fibril orientation).

The response of PMN to alkali-degraded corneal samples in this CG-VCA system may mimic the early PMN response to chemoattractants generated after an alkali injury of the corneal tissue. In the alkali-injured cornea, the first wave of PMN infiltration occurs within hours. This infiltration reaches a peak in 2–3 days, without ulceration, followed by a more intense infiltration beginning approximately 7 days after the injury and peaking during the period when the ulcers develop. Evidence for this diffusibility rests with our own experimental data, which shows that PMN rapidly respond to this low molecular weight chemoattractant in a collagen gel matrix.

In a study made after our original report of the discovery of a PMN locomotion-inducing agent produced from alkali-degraded corneas, other investigators reported the discovery of a > 100,000 Dalton chemoattractant obtained by alkali treatment of an inverted corneoscleral cup. The checkerboard analysis of Boyden chambers (done in these experiments to measure chemotaxis) is suspect in its prediction of events in vivo. The reliability of cell movement in Boyden chambers is influenced by the assumptions made on the degree of adherence to artificial substrates and the lack of direct visual tracking. Although these chambers have been useful in the past for identifying possible chemotactic responses, the technology used alone is being supplanted.

The use of the CG-VCA system, reported in this article, avoids the basic theoretic assumptions and allows for direct visualization and quantitation of the movement of cells subjected to a chemoattractant gradient in a more physiologic, collagen gel matrix. In addition, a broad effect on the behavior of PMN locomotion was defined. This computer-assisted tracking system demonstrates that the alkali-generated chemoattractant induces an unequivocal chemotactic effect on PMN.

The chemoattractant mediator of PMN is of substantial importance in furthering our treatment of alkali injuries. Previous studies have shown that PMN can be inhibited from entry into the cornea after alkali injury by citrate treatment and that such treatment reduces the incidence of corneal ulcers. Although this treatment approach is effective, equally important studies are underway to characterize this chemoattractant and identify potential inhibitors.

| HBSS control | 18.1 | 39.3 | 5.2 | 48.5 | 19.8 | 10.6 |
| 24-hr alkali | 1:21 | 66.7* | 17.5* | 8.8 | 93.9* | 40.6 | 10.9 |
| 1:14 | 72.0* | 12.3* | 9.3* | 95.8* | 44.1 | 10.3 |
| 1:7 | 72.0* | 14.5* | 9.9* | 106.6* | 54.0* | 10.6 |
| Undiluted | 70.1* | 15.4* | 9.6* | 104.0* | 43.6 | 10.8 |
| 30 min alkali | 1:21 | 38.5 | 38.4 | 4.5 | 40.9 | 9.3 | 10.2 |
| 1:7 | 62.5* | 21.3* | 7.4 | 76.6 | 35.8 | 10.2 |

Values are presented as adjusted means from a balanced incomplete block ANOVA.
* Significant at P ≤ 0.05 level versus control by the Dunnett’s test.
PMN Chemoattractant From Alkali-Degraded Cornea

Key Words
alkali injury, neutrophil chemoattractant, polymorphonuclear clear leukocyte, chemotaxis, collagen gel, visual chemotaxis assay

Acknowledgments
The authors thank Dr. David Hurst (Professor of Biostatistics, The University of Alabama at Birmingham) for the statistical design and analysis in these experiments.

References

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