The Effects of Citrate on the Adherence of Neutrophils to Nylon Fibers In Vitro

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The therapeutic effect of citrate on alkali-burned rabbit eyes has been shown to significantly decrease the frequency and severity of corneal ulcerations.1,2 Pfister et al suggested that the therapeutic effect of citrate might be related to its inhibition of polymorphonuclear leukocytes (PMNs).3-5 PMNs are the predominant inflammatory cell in alkali-burned corneas and apparently play a major role in the development of ulcerations.6-8 Prior to accumulation in these corneas, PMNs must attach to the vascular endothelium of preexisting limbal or conjunctival vessels or to new vessels forming after the injury. Without the adherence process PMNs would remain in the circulatory system, unable to reach the injury site.

The mediators involved in the augmentation of adherence in alkali-burned eyes are unknown. Leukotriene B4 (LTB4) is produced by PMNs as a product of arachidonic acid metabolism. It is known to augment PMN adherence in vitro9 and in vivo.10 Elevated levels of LTB4 occur in alkali-burned eyes.11 Alkali-burned collagen breakdown product(s) have been suggested to be a possible mediator of PMN activity in the burned cornea.12 There are many other mediators potentially involved in the inflammatory response to eye injury.

The purpose of this study was to determine the effects of citrate on the adherence of resting and LTB4-treated PMNs in vitro. Ascorbate was tested in this system because of its favorable effect in alkali-burned eyes.13 Alkali-burned collagen was evaluated for its potential as an adherence augmentor. Nylon fibers were used as a surface for adherence, having previously been demonstrated to produce results similar to vascular endothelial cell cultures.14

Materials and Methods

Materials

Trisodium citrate, sodium ascorbate, bovine serum albumin (BSA), CaCl2, MgCl2, and Type I bovine collagen were purchased from Sigma Chemical Co. (St. Louis, MO). LTB4 was a gift from Merck Frosst Canada Inc. (Quebec, Canada). Methanol and NaOH were obtained from Baker Chemical Co. (Philipsburg, NJ) and Fisher Scientific Co. (Fair Lawn, NJ), respectively. Hanks balanced salt solution (HBSS) was purchased from Gibco Laboratories (Chagrin Falls, OH).
PMN Isolation

Polymorphonuclear leukocytes were isolated according to a previous paper. The cell suspension was greater than 80% PMNs (96–99% viability) with the remaining percentage consisting of red blood cells and less than 5% platelets, lymphocytes, and eosinophils.

Adherence Assay

Attachment of PMNs to nylon fibers was determined using a modification of the adherence assay described by MacGregor et al. Briefly, 1 cc tuberculin syringes were uniformly packed with 65 mg (40 mg from certain collagen treated samples as specified) of scrubbed nylon fiber (3 Denier, 3.81 cm, Type 200, Fenwal Laboratories, Morton Grove, IL) to a volume of 0.4 cc (Fig. 1). All stock solutions and PMN suspensions were prewarmed to 37°C for 5 min. Each test substance (citrate, Ca2+ and Mg2+, ascorbate, LTB4, and burned or salt-treated collagen) was preincubated with PMNs for 2 min (37°C) unless otherwise specified. All incubation suspensions contained the following final concentrations: 500 μM Ca2+, 600 μM Mg2+, and 2% BSA (certain collagen-treated samples contain no BSA as noted). One milliliter aliquots of the incubation suspension were added to each tuberculin syringe and allowed to flow through the fibers for 2 min with the effluent collected in a polypropylene test tube. Percent PMN adherence was calculated based on cell counts performed before and after the cell suspensions were passed through the syringes. Cell counts were determined by the microcellcounter model CC-130 (Sysmex, Toa Medical Electronics, Los Alamitos, CA). All incubations were maintained at a pH of 7.2 to 7.6 and an osmolality of 270 mOsm to 330 mOsm.

LDH Assay

The percentage of cell death was determined by measuring the activity of lactate dehydrogenase (LDH) in the supernatant after centrifugation of each sample. LDH release for all experiments was less than 5% of the total.

Statistics

The data were analyzed for statistical significance by the student t-test.

Results

LTB4 significantly increased PMN adherence above unaugmented control levels (Fig. 2). Pretreatment of PMNs with 12 mM citrate significantly inhibited this adherence to well below even the control
values. When Ca$^{2+}$ and Mg$^{2+}$ were added to the suspension with citrate there was complete reversal of the inhibitory effect of citrate back to the augmented levels.

Inhibition of PMNs by citrate pretreatment followed a dose-response curve. Resting PMNs pre-treated with increasing concentrations of citrate reduced adherence from 35% to 10% (Fig. 3). Further reductions in adherence could not be achieved above 8 mM citrate. Increasing concentrations of citrate reduced LTB$_4$-augmented PMN adherence from 65% to 15% (Fig. 4).

Pretreatment of resting PMNs with increasing concentrations of ascorbate showed some reduction in adherence but this was not statistically significant even at unphysiologically high levels (Fig. 5). No significant change in LTB$_4$ augmentation of PMN adherence occurred in the presence of increasing concentrations of ascorbate (Fig. 6).

Burned or NaCl-treated collagen incubations containing BSA had no effect on the adherence of resting or LTB$_4$-treated PMNs even when preincubated for up to 60 min (Fig. 7). This prolonged treatment of PMN with burned collagen in the presence of BSA did not increase the release of LDH (60 min collagen pretreatment of resting PMN, $\bar{x} = 0.1 \pm 0.1\%$ of total LDH, $n = 4$; control, $\bar{x} = 0.4 \pm 0.5\%$ of total LDH, $n = 5$). The addition of supernatant from alkali-burned collagen produced a strong inhibition of the adherence of resting or LTB$_4$-treated PMNs when BSA was omitted from the samples (Fig. 8). This inhibition increased from a collagen dilution of 1:1000 to 1:100, plateauing at 1:10. Control studies of collagen treated with NaCl alone (1:10) inhibited the adherence of resting PMNs by 35.0%.

Cell counts taken before and after preincubation with each test substance (LTB$_4$, citrate, Ca$^{2+}$ and Mg$^{2+}$, ascorbate, and burned or salt-treated collagen), but before nylon fiber passage, were not statistically different (ie, before LTB$_4$ addition = 6.7 ± 0.2 × 10$^6$ PMNs/ml, after LTB$_4$ addition = 6.5 ± 0.2 × 10$^6$ PMNs/ml).

**Discussion**

Cellular inflammatory reactions are thought to begin with the release of adherence augmenting factors such as LTB$_4$ and C5a. These substances induce the attachment of PMNs to vascular endothelia, favoring diapedesis. Extravascular PMNs then move along a chemoattractant gradient to the affected tissue where they accumulate. Metabolic stimulation of these cells in the injured tissues causes a biochemical shift to enhanced oxygen utilization, superoxide radical production, phagocytosis and the release of lysosomal enzymes. The process of PMN adherence is therefore critical to the initiation of an inflammatory reaction.

The adherence process has previously been reported to be inhibited by the chelation of divalent cations. PMNs did not adhere to traumatized endothelium, in vivo, when the concentration of divalent cations was reduced by chelation with systemically or locally applied ethylenediamine tetraacetic acid (EDTA) or citrate.
(EDTA)\textsuperscript{19,20} EDTA, oxalate and sodium citrate also prevented PMN adherence to glass beads in the presence of heparin.\textsuperscript{21} The importance of divalent cations in this process is further emphasized by the reduction of in vivo and in vitro adherence induced by local anesthetics which are thought to displace cell membrane calcium.\textsuperscript{22,23}

The reversal of citrate inhibition of adherence by divalent cations shows that the adherence of PMNs to nylon fibers is significantly controlled by the concentration of cations in the media. Citrate has previously been shown to inhibit the locomotion and metabolic stimulation of PMNs, including the respiratory burst, phagocytosis and degranulation.\textsuperscript{35} The cationic requirements of specific mediators may determine the actual degree of inhibition of chelators such as citrate in each individual case.

The affinity of PMNs to adhere to artificial materials has been used to isolate these cells. PMNs attached to glass or nylon fibers can be partially removed by washing with solutions of EDTA, oxalate, or acid citrate dextrose (ACD).\textsuperscript{24-26} This property of ACD is useful in filtration leukapheresis for the separation of PMNs by reversing cell attachment to nylon fiber columns.\textsuperscript{27} This procedure is based on the detachment of PMNs already adhered and not the prevention of adherence. ACD is slightly acidic (pH 6.5 to 6.8) and contains dextrose while the plasma normally used in this procedure contains heparin, making it difficult to make an exact comparison to the studies presented here. Our paper is the first time pretreatment with sodium citrate has been shown to inhibit an augmented PMN adherence.

Other anti-inflammatory substances have been tested for their effects on PMN adherence.\textsuperscript{28} Glucocorticoids had no effect on adherence in vitro while inhibiting adherence in vivo by about 35%. This difference was resolved with the finding that plasma from subjects given glucocorticoids contained a factor which inhibited PMN adherence in vitro (38.4%). This moderate effect produced indirectly by glucocorticoids is in marked contrast to the strong inhibition of adherence by citrate noted in this study. Furthermore, there are dose-dependent complications of steroids when used topically, including interference with wound healing,\textsuperscript{29} cataract formation,\textsuperscript{30} and the deeper and more severe spread of ocular herpetic disease.\textsuperscript{31} The effect of citrate on PMN adherence, in vivo, is currently under investigation.
Fig. 7. Supernatant from alkali-burned Sigma collagen did not significantly alter the adherence of resting or LTB4 (1 × 10^-7 M)-treated PMNs (2 min preincubation) in the presence of 2% BSA; 65 mg of packed nylon fibers were used. Adherence is expressed as the mean ± SEM of seven or nine triplicate incubations. A vs. B, C, D, or E = not significant; a vs. b or c = 0.001 < P < 0.01; b vs. c = not significant. Resting PMNs were also preincubated with burned collagen (1:10 final dilution) for 15, 30, and 60 min with no significant effect on the percent adherence (15 min collagen x = 27.7 ± 1.3, control x = 26.2 ± 1.4; 30 min collagen x = 30.8 ± 3.5, control x = 27.6 ± 3.2; 60 min collagen x = 32.6 ± 4.0, control x = 27.6 ± 3.2; nine triplicate incubations for each sample). LTB4-treated PMNs were preincubated with burned collagen (1:10 final dilution) for 60 min with no significant effect on the percent adherence (60 min collagen x = 46.7 ± 2.9, LTB4 x = 49.5 ± 3.2; eight triplicate incubations for each sample). An additional control sample was tested by subjecting resting PMNs to NaCl-treated collagen which was diluted 1:10 with HBSS in the final incubation suspension (control, x = 23.7 ± 8.1; NaCl treated collagen control, x = 23.4 ± 1.8).

The results of the present experiment suggest that the inhibition of PMN adherence to the vascular endothelia by topical citrate might partially explain its favorable effect in the alkali burned eye. The application of 10% citrate drops (512 mM) immediately after alkali injury to the rabbit eye, significantly decreased the incidence of corneal ulcers.12 This is caused by tissue levels of citrate which rise appreciably in conjunctiva, episclera, cornea and aqueous humor. Evidence for this is our prior work showing an average concentration of citrate of 8 mM in the cornea 30 min after dropping.2 Considering the extremely high concentration of citrate used, there is probably a very steep gradient from the anterior to the posterior layers. This may be deduced by the 1 to 2 mM concentrations of citrate noted in the aqueous humor at the same time that citrate levels of up to 8 mM are present in the cornea. Given these studies it is reasonable to assume that the conjunctival vessels and perivascular arcades remaining after alkali injury would be bathed by levels of citrate sufficient to inhibit PMN adherence to the vascular endothelia. Whether this inhibitory effect of citrate is on the PMN, vascular endothelium, or both, is unknown.

When citrate treatment was applied subcutaneously in rabbits there was a significant reduction in the severity of ulceration but only a trend toward reduced incidence of ulceration.3 This result occurred with plasma citrate levels peaking at 1.7 mM, similar to the concentration range observed for inhibition of LTB4-augmented adherence of PMNs to nylon fiber columns. These levels of plasma citrate in humans are associated with moderate side effects, possibly limiting the efficacy of systemically administered citrate. These results suggest that citrate might inhibit PMNs intravascularly, thus reducing the numbers of PMNs escaping into the target tissues. Indeed, citrate
has been reported to reduce PMN accumulation in ocular tissues during the inflammatory response associated with intravitreal endotoxin injection.\textsuperscript{32} If PMNs can be prevented from entering the target tissue (cornea), then the ulcerative process might be stopped.

Reduced PMN adherence to the vascular endothelium would lead to fewer PMNs in injured tissue. This reduction in inflammatory cells might lead to a reduction in the amount or type of inflammatory mediators produced directly or indirectly by these cells. Borodic et al have recently determined that LT\textsubscript{B\textscript{4}} and other arachidonic acid metabolic products produced by PMNs are greatly increased in alkali-injured eyes at about the time of corneal ulceration.\textsuperscript{11}

There are a variety of other mediators which may be produced indirectly by PMNs at the injury site, including C5a\textsuperscript{33} and superoxide radical-lipid-serum albumin complexes.\textsuperscript{34} Citrate might lessen the inflammatory reaction by inhibiting adherence, thus reducing the production of inflammatory mediators at the injury site.

Alkali-treated collagen was evaluated for its potential as an adherence augmenter for PMNs because previous work demonstrated its potential as a locomotory agent.\textsuperscript{12,35} The more concentrated dilutions of burned collagen and, to a lesser extent, sodium chloride-treated collagen inhibited the adherence of resting or LT\textsubscript{B\textscript{4}}-treated PMNs when BSA was absent. These findings support our previous observation that burned collagen supernatants prevented PMNs from adhering to glass slides when BSA was absent.\textsuperscript{12} In contrast, when BSA is present, burned collagen has no effect on the adherence of resting or LT\textsubscript{B\textscript{4}}-treated PMNs. Since serum albumin is always present when PMN adherence to vascular endothelium occurs, it is unlikely that alkali-injured collagen plays any role in the interaction of PMNs to endothelium. BSA does appear to exert a protective effect on PMNs by preventing cell lysis after prolonged exposure to burned collagen supernatant. In this regard, the presence or absence of serum albumin (SA) may play an important role in the burned cornea where the concentration of SA may be reduced.

It has been suggested that exogenous ascorbate might act as a superoxide radical scavenger in inflammatory eye disease, reducing ocular tissue destruction in the process.\textsuperscript{36} Similarly, Williams and Paterson suggested that ascorbate might act as an endogenous anti-inflammatory agent in the eye by reacting with oxygen radicals and/or metabolites generated by PMNs, likewise diminishing damage to intraocular tissues.\textsuperscript{37,38} On the other hand, ascorbate might be detrimental in some inflammatory situations by actually enhancing certain PMN activities.\textsuperscript{39-41} Finally, some studies have shown that ascorbate has no effect on certain PMN functions.\textsuperscript{42,3} The present study determined that ascorbate has no significant effect on the adherence of resting or LT\textsubscript{B\textscript{4}}-treated PMNs. The usefulness of exogenous ascorbate as an anti-inflammatory agent needs further study.

**Key words:** polymorphonuclear leukocytes, vascular, adherence, citrate, leukotriene B\textsubscript{4}, collagen, ascorbate

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**References**


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