Polymorphonuclear Leukocytic Inhibition by Citrate, Other Metal Chelators, and Trifluoperazine

Evidence to Support Calcium Binding Protein Involvement

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Corneal ulceration after severe alkali burns of the eye is thought to result principally from collagen breakdown as a consequence of local polymorphonuclear leukocyte (PMN) activities. The favorable effect of topical citrate on such burns suggested a direct effect on these inflammatory cells. These in vitro studies show that the stimulation of human PMN by opsonized zymosan can be inhibited by citrate, EDTA, and EGTA. These compounds interfere with opsonized zymosan attachment to PMN, preventing the respiratory burst, phagocytosis, and degranulation. Reversal of this inhibition by calcium and/or magnesium suggests the mechanism is calcium chelation. Trifluoperazine (TFP) inhibition of opsonized zymosan attachment and phagocytosis implicates the involvement of calmodulin. We propose that citrate, EDTA, and EGTA interfere with the receptor mediated attachment of opsonized zymosan to the PMN cell membrane, leaving the PMN in a resting, granulated state. Inhibition of the receptor system by calcium depletion may be the result of interference with calcium-calmodulin modulated microfilament and/or microtubule interfaces in the PMN plasma membrane. It is postulated that comparable events occur in the citrate treated alkali burned cornea. Citrate inhibition of PMN may be useful in other eye and systemic diseases. Invest Ophthalmol Vis Sci 25:955-970, 1984

Both topical sodium citrate and sodium ascorbate treatment has been shown to reduce the incidence of corneal ulceration and perforation following certain experimental alkali burns in the rabbit. However, citrate but not ascorbate was effective even in the treatment of very severe alkali burns. In the case of the very severely burned corneas, the population of fibroblasts was sparse and the major cell type present, up to 2 weeks after the burn, appeared to be polymorphonuclear leukocytes (PMNs) (Pfister and Haddox, unpublished data). This led us to think that citrate might be in some way acting upon the PMN to inhibit the ulceration process, perhaps by interfering with calcium levels in the environment since citrate is a known calcium chelator.

The invasion and subsequent activity of PMNs have been implicated as a major cause for corneal ulceration in human and animals. PMNs chemically attracted into the damaged corneal tissue become stimulated to phagocytize and degranulate. The process of degranulation releases into the cornea a variety of enzymes, including collagenase, which is thought to be primarily responsible for ulcer development.

The vigorous biochemical activity associated with PMN degranulation and phagocytosis requires a substantial amount of cellular energy. These energy requirements are met by a respiratory burst in the PMN, achieved by partially shifting energy metabolism from the glycolysis pathway to the hexosemonophosphate shunt (HMP). In the resting PMN, the HMP accounts for only about 1% of energy production increasing to some 30% in the activated PMN. This enhanced oxygen utilization by the PMN also results in the production of free oxygen radicals and hydrogen peroxide, which is released into the intracellular and extracellular environment.

The purpose of this study was to examine the influence of citrate and other compounds upon the biochemical activity of PMNs in vitro. PMNs were examined in their resting state and also following stimulation by opsonized zymosan. We examined oxygen utilization, enzyme release, ultrastructure, and attachment and phagocytosis of zymosan.

Materials and Methods

PMN Isolation

Following the technique of Ferrante and Thong, 7 ml of fresh human whole blood, taken by venipuncture...
in heparinized tubes, were layered on 3 ml of Hypaque–Ficoll (density 1.119) and centrifuged at 200 × g for 20–25 min at room temperature. A diffuse band of PMN between the red blood cell (RBC) pellet and the compact mononuclear cell band was removed and pelleted by recentrifugation at 400 × g for 30 min. Resuspension in Hanks buffered salt solution (HBSS) containing 500 μM Ca2+ and 600 μM Mg2+ yielded a PMN suspension 98–99% pure with respect to leucocytes (96–99% viability) and an equal number of RBCs present with less than 5% platelets. When ammonium chloride (0.82%) or NaCl (0.2% followed by 1.6%) were used to lyse the remaining RBCs, as recommended by some authors, significant effects on PMN morphology were visible by light microscopy. Aguado et al.7 purified PMN through dextran sedimentation followed by ammonium chloride lysis and observed very similar results (membrane alterations, increased numbers of cellular vacuoles and decreased levels of cellular enzymes). Since RBCs cannot undergo a respiratory burst, attach or phagocytize opsonized zymosan, or release β-glucuronidase and cannot be attached or phagocytized by PMN unless opsonized, it was considered wisest to preserve PMN integrity by avoiding these lytic effects. When critical experiments were repeated using PMN suspensions free of RBCs present with less than 5% platelets. When ammonium chloride (0.82%) or NaCl (0.2% followed by 1.6%) were used to lyse the remaining RBCs, as recommended by some authors, significant effects on PMN morphology were visible by light microscopy. Aguado et al.7 purified PMN through dextran sedimentation followed by ammonium chloride lysis and observed very similar results (membrane alterations, increased numbers of cellular vacuoles and decreased levels of cellular enzymes). Since RBCs cannot undergo a respiratory burst, attach or phagocytize opsonized zymosan, or release β-glucuronidase and cannot be attached or phagocytized by PMN unless opsonized, it was considered wisest to preserve PMN integrity by avoiding these lytic effects. When critical experiments were repeated using PMN suspensions free of RBCs (ammonium chloride or NaCl lysis) the results were consistent with the suspensions containing RBCs.

Opsonization

Human whole blood was obtained by venipuncture and allowed to clot in sterile tubes at room temperature for no longer than 90 min. Centrifugation for 30 min at 300 × g provided serum held in 2-ml aliquots at −20°C until used in the following 5 days. Zymosan (Sigma) was opsonized by incubating 10 mg/ml in type AB serum for 30 min at 37°C. Fresh opsonized zymosan was prepared every 4 hr.

Internal controls were performed to show that none of the chelators had any deleterious effect on the products of the opsonization process as judged by the capacity of chelator treated zymosan to stimulate PMN.

Respiratory Burst

Oxygen utilization by PMN (4.5 × 10⁶ cells/ml) was measured by a Clark-type oxygen monitor (YSI Model 53) in an incubation chamber maintained at 37°C in a pH range of 7.2–7.8. The appropriate study solution (citrate, EDTA, EGTA or TFP) was added to the PMN suspension in the incubation chamber 5 min prior to zymosan stimulation. In some cases TFP was used immediately before stimulation. Some incubations were performed to determine if cations could reverse chelor induced inhibition. In these cases, additional cations were added following preincubation with the inhibitor but immediately prior to zymosan stimulation. Stimulation of PMN was achieved by the addition of 100 μl of opsonized zymosan in serum for a total chamber volume of 3 ml.

In a separate group of experiments, the osmolality of the incubation mixture was varied by adding sucrose or sodium chloride. The respiratory burst, enzyme release, and phagocytosis were inhibited by solutions of greater than 375 mOsm but not by solutions as low as 250 mOsm. Our experiments were conducted with solutions varying from 280 mOsm to 320 mOsm.

Enzyme Assays

The release of β-glucuronidase (β-gluc) from PMN was determined in the supernatant, after centrifugation of the incubation mixture, by a modification (18-hr incubation) of the technique of Talalay et al.8 To evaluate cell viability, lactic dehydrogenase (LDH) activity9,10 was measured in these same supernatants. LDH release in the citrate, EDTA, and EGTA pretreated samples as well as all cation reversal samples was less than their mean zymosan stimulated samples. Total LDH and β-gluc enzyme release from the same batch of cells was obtained individually after cell lysis by Triton X-100 (0.2%) for 2 hr at 37°C. Control experiments utilizing pure RBC suspensions determined that, in our system, PMN contained 100% and 95% of the measureable β-gluc and LDH activity, respectively.

Attachment and Phagocytic Study

The attachment and phagocytosis of opsonized zymosan by PMN was followed in an incubation chamber under identical conditions as the oxygen monitoring studies. The reaction was stopped by adding 3 ml of 4% glutaraldehyde at 15 sec or 60 sec. The presence of zymosan attachment or phagocytosis was identified in 100 cells examined under oil immersion. Phagocytic activity was compared with the respiratory burst and enzyme release from the same sample at the end of 10 min of zymosan stimulation by counting the total number of zymosan particles phagocytized. Briefly, the total number of particles found in 100 cells...
subjected to the test compound was expressed as a percentage of the total number of particles inside 100 PMN in the zymosan stimulated control group. PMN found in clumps were not counted if their cellular contents were not clearly visible.

Some studies were performed in the cold (0-4°C) for 10 min and the total number of attached zymosan particles was expressed as a percentage of the total number of zymosan particles phagocytized at 37°C.

Unopsonized zymosan is phagocytized by PMN only when brought into close contact by centrifugation. To study the influence of the test solutions on this process unopsonized zymosan (10 mg/ml) was suspended in HBSS with 2 mM Ca²⁺ and 1 mM Mg²⁺ and incubated for 30 min at 37°C. Pretreated (12 mM Citrate, 1.5 mM EDTA, 4.0 mM EGTA, and 40 μm TFP) or control PMN suspensions, following the addition and mixing of unopsonized zymosan (100 μl), were centrifuged to a pellet (600 × g for 1 min at room temperature) and allowed to incubate for 1 min before glutaraldehyde fixation. Microscopic observation determined the percentage of cells resting, phagocytizing, or with attached particles only. Appropriate controls determined that virtually no zymosan attachment or phagocytosis occurred during the suspension phase.

Electron Microscopy

At the end of some of the respiratory burst experiments, 3 ml of 2% glutaraldehyde in 0.1 M cacodylate buffer (pH, 7.3) was added to the incubation chamber. The fixed cells were pelleted by centrifugation, postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH, 7.3), dehydrated in ethanol, and embedded in Poly/Bed 812. Sections were stained with uranyl acetate and lead citrate. Specimens were examined in a Phillips 301 transmission electron microscope.

Morphometry of granule volume was performed on PMN pellets obtained from three replicate experiments. PMN were stained for myeloperoxidase to distinguish azurophilic granules (positive) from specific granules (negative). The results were expressed as a percentage of cytoplasmic volume (excluding nuclei and phagocytic vacuoles). A 1 cm² lattice grid was superimposed over 15 to 20 micrographs of PMNs (at least 1500 points per treatment) printed to a final magnification of 36,000 for each treatment. This technique, elaborated by Hoffstein et al., employs point counting of intersections falling on azurophilic granules, specific granules or cytoplasm.

Preparation of Solutions

Trisodium citrate and sodium ascorbate (gifts from Hoffman-LaRoche) solutions were prepared in appropriate concentrations in distilled water at a pH of 7.4 by titration with HCl. Disodium edetic acid (pH, 8.7-9.0) and EGTA (pH, 7.8-8.5) solutions were prepared in an equal concentration of Tris buffer (to correct for the pH drop caused by the binding reaction). For Ca²⁺ reversal of EGTA, equimolar Tris buffer was required in the stock CaCl₂ solution (pH = 9.0) to correct for an additional pH drop. Ca²⁺ (CaCl₂) and Mg²⁺ (MgCl₂), used for all other reversal experiments, were prepared in distilled water at appropriate concentrations with a pH of 7.4. Trifluoperazine (a gift from Smith, Kline, and French) was made up in Hanks buffered salt solution (cation free) at pH 6.6 by titrating with NaOH. All solutions were made fresh each day.

Statistics

All statistical evaluations were performed with the Student's t-test. Standard error of the mean is employed throughout the paper.

Results

Oxygen Utilization (Respiratory Burst), β-Glucuronidase Release, Phagocytosis, and the Ultrastructure of Resting and Stimulated PMN

Resting PMN are small rounded cells exhibiting a low level of O₂ utilization (Fig. 1A) and minimal enzyme release (β-gluc: control = 2.6% ± 0.3% of total enzyme, N = 63). No evidence of phagocytosis is seen in resting PMN. The cytoplasm of resting PMN con-
Fig. 1. B, Electron micrograph of unstimulated PMN. Resting PMN were suspended in HBSS containing 500 \( \mu \text{m} \) Ca\(^{2+} \) and 600 \( \mu \text{m} \) Mg\(^{2+} \). These PMN, stained for myeloperoxidase in the azurophilic granules (A) are rounded and nonpolar because serum is absent from the media (n = nucleus and s = specific granule).
Fig. 1. C, Electron microscopy of stimulated PMN. PMN shown are stimulated by the addition of opsonized zymosan (oz). The dense contents of some azurophilic granules (a), stained for myeloperoxidase, have been released into the phagocytic vacuoles (arrows) while some also spills directly into the extracellular media (n = nucleus and s = specific granule).
contains large numbers of specific and azurophilic granules (Fig. 1B). When the PMN were stimulated in the presence of opsonized zymosan, oxygen utilization increased dramatically (Fig. 1A) and enzymes were released from the granules (β-gluc: stimulated = 18.4% ± 0.5% of total enzyme, n = 62).

In stimulated PMN, filopodia envelope the opsonized zymosan particles. At the same time some granules...
Fig. 2. PMN were pretreated with 12 mM citrate for 5 min. Because of the addition of serum with opsonized zymosan, PMN develop filopodia but do not show attachment or phagocytosis of zymosan. These PMN are not different from unstimulated control PMN to which zymosan treated serum was added (n = nucleus, a = azurophilic granule, and s = specific granule).
The respiratory burst of PMN is inhibited by pretreatment with citrate, EDTA, or EGTA, in increasing order (Fig. 2A, B, C). When the Ca\(^{2+}\) and/or Mg\(^{2+}\) concentration of the incubation medium is decreased, then less chelator compound is required for PMN inhibition.

Phagocytosis decreases dramatically as the concentrations of citrate, EDTA, or EGTA increases in the incubation media. The decrease in the total number of phagocytosed zymosan particles in PMN pretreated with citrate, EDTA, or EDTA parallels the percentage of inhibition of the respiratory burst (Fig. 2A, B, C).

Figure 2A, B, C shows that \(\beta\)-gluc release from pretreated PMN is reduced progressively as the concentrations of citrate, EDTA, and EGTA is increased. Inhibition of enzyme release parallels, but is generally below, the curve showing inhibition of the respiratory burst.

The ultrastructural appearance of PMN, inhibited by citrate, EDTA, or EGTA, is indistinguishable from that of resting PMN (Fig. 2D). The small remaining number of PMN phagocytizing even in the presence of these compounds do so in a manner similar to the stimulated PMN.

Calcium and Magnesium Reversal of Citrate, EDTA, and EGTA Inhibition

The inhibition of the respiratory burst effected by all three cation chelators (citrate, EDTA, and EGTA) was reversible by adding extracellular Ca\(^{2+}\) and/or Mg\(^{2+}\) to the medium. Ca\(^{2+}\), or Mg\(^{2+}\), added separately, partially reversed citrate-induced inhibition while combinations of these two cations produced the greatest reversal. Total reversal of the inhibition occurred when both cations were present at concentrations of 1 mM Ca\(^{2+}\) and 2 mM Mg\(^{2+}\) (Fig. 3A). Inhibition induced by EDTA and EGTA was totally reversed by Ca\(^{2+}\).

Phagocytosis was stimulated by the addition of 1, 4, and 8 mM Mg\(^{2+}\) to 52%, 74%, and 84% stimulation, respectively. Combinations of cations produced the following reversals of phagocytosis: 1 mM Ca\(^{2+}\) + 1 mM Mg\(^{2+}\) = 69% stimulation, 1 mM Ca\(^{2+}\) + 4 mM Mg\(^{2+}\) = 92% stimulation, and 1 mM Mg\(^{2+}\) + 4 mM Ca\(^{2+}\) = 80% stimulation.
alone; a lesser reversal of EDTA occurred with Mg\textsuperscript{2+} (Figs. 4, 5). A very small reversal of EGTA inhibition was effected by Mg\textsuperscript{2+}.

When the inhibition by chelators is reversed by Ca\textsuperscript{2+} or Mg\textsuperscript{2+}, there is a significantly greater \(\beta\)-gluc release (Figs. 3B, 4, 5). This reversal closely parallels the respiratory burst reversal. Although Mg\textsuperscript{2+} reverses citrate inhibition more effectively at lower concentrations, Ca\textsuperscript{2+} and Mg\textsuperscript{2+} have equal effects at the 8 mM level. The reversal of EDTA inhibition of \(\beta\)-gluc release is very effective with Ca\textsuperscript{2+} or Mg\textsuperscript{2+}, the former being 100% at the highest concentration (Fig. 4). EGTA inhibition is reversed totally by Ca\textsuperscript{2+} but only slightly by Mg\textsuperscript{2+} (Fig. 5).

Chelator inhibition of phagocytosis is reversed by Ca\textsuperscript{2+} and/or Mg\textsuperscript{2+}. Only Mg\textsuperscript{2+} reversal of EGTA inhibition is poor.

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**Trifluoperazine (TFP)**

TFP, a potent inhibitor of Ca\textsuperscript{2+-}calmodulin, decreased the respiratory burst in a linear fashion when used in concentrations from 20 \(\mu\)M to 40 \(\mu\)M (Fig. 6). When TFP was added to PMN 5 min before the addition of zymosan, there was a delay in the beginning of a partially inhibited respiratory burst, which increased with higher concentrations. When TFP was added immediately prior to zymosan stimulation, no lag period was observed. It is important to note that 5-min pretreatment with 40 \(\mu\)M TFP caused a significant release of LDH (control TFP = 17.4% ± 0.1% of total; stimulated TFP = 21.3% ± 3.6% of total), while LDH release after immediate treatment was consistently lower (control TFP = 8.9% ± 2.8% of total; stimulated TFP = 14.9% ± 1.8% of total), but still higher than unstimulated or zymosan stimulated controls without TFP addition (unstimulated control = 2.5% ± 0.6% of total; stimulated control = 8.7% ± 1.0% of total).
Fig. 6. The influence of TFP (immediate, no preincubation) on respiratory burst, $\beta$-gluc release, and phagocytosis of PMN. The mean unstimulated control and zymosan stimulated values are represented by 100% and 0% inhibition, respectively. TFP was added immediately prior to the addition of opsonized zymosan. The $\beta$-gluc curve was corrected for nonspecific $\beta$-gluc release (cell death) by subtracting the enzyme released from the appropriate unstimulated control TFP incubations (20, 30, and 40 $\mu$m).

The inhibition of $\beta$-gluc release by TFP (corrected by subtracting the $\beta$-gluc release from unstimulated control TFP incubations) was generally less than that noted with the respiratory burst for both 5 min pretreatment and immediate pretreatment. Figure 6 shows that the inhibition of phagocytosis effected by TFP was similar to the inhibition of the $\beta$-gluc release.

Even in the resting state, 5-min pretreatment with 40 $\mu$m TFP appears to have an effect on the PMN plasma membrane commonly characterized by a shallow depression in one side of an otherwise smooth, rounded cell. In those few cells phagocytizing in the presence of TFP, zymosan particles appear to "sink" into the cell with or without the presence of rudimentary filopodia. In these stimulated cells, there appears to be a substantial reduction in the numbers of microtubules.

Attachment and Phagocytosis

Pretreatment with citrate, EDTA, and EGTA dramatically reduced the attachment of opsonized zymosan particles to PMN 15 sec (and 60 sec) after stimulation (Figs. 7A, B, C). The inhibition induced by all three chelators was reversed by adding Ca$^{2+}$ and/or Mg$^{2+}$ immediately before stimulation.

When PMN were treated with citrate or EGTA 15 sec after stimulation, but continued to be incubated for a total of 60 sec, in the presence of the chelator, the percentage of cells phagocytizing were statistically similar to the percentage of cells with particles attached after 15 sec (Figs. 8A, C). Only a few additional cells showed attachment after exposure in both cases. When EDTA was added to the incubation mixture 15 sec after stimulation, only about one-half of the particles attached after 15 sec were phagocytized after 60 sec (Fig. 8B). This delay of the phagocytic process was further demonstrated by incomplete particle ingestion at this stage.

Pretreatment with 40 $\mu$m TFP (both 5 min and immediate) inhibited zymosan attachment at 15 sec as seen in Figure 9. A 3–6-min lag period before phagocytosis began in the 5 min-pretreatment group coincided with the delay in the initiation of the respiratory burst. Thereafter, attachment and phagocytosis proceeded at a substantially reduced rate compared with controls. Although the majority of PMN treated with TFP had attached and/or phagocytized particles at 12 min, the number of particles per cell was significantly reduced ($P < 0.001$) from zymosan stimulated control (zymosan = 899.4 ± 49.3/100 cells; TFP = 399.9 ± 100.5/100 cells).

Opsonized zymosan particle attachment to PMN was almost abolished when the incubation temperature was lowered from 37°C to 0–4°C. Zymosan attachment to PMN at 0–4°C was 0.7 ± 0.4, (N = 7) compared with 335.4 ± 31.0 (N = 7) phagocytized particles at 37°C (99.8% inhibition).

The effects of citrate, EDTA, EGTA, and TFP on PMN phagocytosis of unopsonized zymosan in a centrifuged pellet is expressed in Table 1. When compared with unopsonized zymosan stimulation, inhibition of phagocytosis was greatest with EDTA and TFP, less with citrate but not significant with EGTA.

Morphometry of PMN Granules

The percentage of PMN cytoplasm occupied by azurophilic or specific granules after incubation is expressed in Table 2. Note that zymosan stimulation
significantly reduces azurophilic and specific granular percentages, while EDTA appears to effectively inhibit most granular release. Citrate, like EGTA and TFP inhibits azurophilic release but only partially inhibits the release of specific granules.

**Ascorbate**

The interference of ascorbate with the measurements of both respiratory burst and β-gluc release prevented its assessment in this system. Phagocytosis of zymosan particles, in the presence of ascorbate (12 mM or 50 mM) was not statistically different from the zymosan stimulated control samples. When compared with zymosan stimulated samples, 12 mM ascorbate was 102.6% ± 5.5% stimulation (n = 6), while 50 mM ascorbate was 101.3% ± 3.2% stimulation (n = 18).

**Discussion**

Citrate inhibits the respiratory burst, phagocytosis, and enzyme release of PMNs by preventing the attachment of opsonized zymosan to the cell membrane. The mechanism of this inhibitory effect is felt to be 

\[ \text{Ca}^{2+} + \text{Mg}^{2+} \]

chelation; a suggestion supported by the inhibitory effects of strong chelators such as EGTA and EDTA. The inhibitory effects of all of these compounds are reversed by the addition of 

\[ \text{Ca}^{2+} \].\]
Fig. 8. The influence of chelators on particle ingestion. The first bar represents the percentage of PMN with attached zymosan particles at 15 sec, while all other bars are percentages (attachment, phagocytosis, or incomplete phagocytosis) after 60 sec of stimulation. Citrate (A), EDTA (B), and EGTA (C) inhibition and cation reversal of particle ingestion.
greater inhibitory effect of EDTA over EGTA is probably related to chelation of Mg\(^{2+}\) as well as Ca\(^{2+}\) by the former but not the latter compound. Our data showing the greater Mg\(^{2+}\) reversal of EDTA inhibited cells compared with EGTA inhibited cells strongly support this contention.

We believe these inhibitory effects of citrate are the consequence of the chelation of Ca\(^{2+}\) and Mg\(^{2+}\) in the extracellular medium and possibly in the PMN plasma membrane. Calcium has been identified in the plasma membrane of resting (unstimulated) PMN, but not in the localized contact area of a cell stimulated by a zymosan particle. Citrate may be removing the membrane stored Ca\(^{2+}\) or preventing the stimulated cell membrane from becoming loaded with Ca\(^{2+}\). In either case, citrate would prevent Ca\(^{2+}\) from accomplishing its primary role as a second messenger and an activator of Ca\(^{2+}\)-binding proteins. This thesis is quite credible now that Ca\(^{2+}\)-calmodulin has been implicated in platelet membrane receptor binding through interaction between microtubules and microfilaments. \(^{20}\) Ca\(^{2+}\)-calmodulin regulates microtubule assembly/disassembly and myosin light-chain kinase. \(^{21,22}\) Furthermore, it has been suggested that local anesthetics affect the mobility of cell surface receptors by displacing membrane bound Ca\(^{2+}\), thus altering the membrane associated microtubule–microfilament system. \(^{23}\) The complete reversal of EGTA and EDTA inhibition of attachment by Ca\(^{2+}\) alone appears to give this cation a major role in the attachment process.

We propose that the inhibitory effects of citrate in this system occur as a consequence of free calcium chelation with subsequent interference with the function of the PMN cell surface receptors. In addition to the chelation of extracellular Ca\(^{2+}\), citrate may remove and chelate calcium held in ionic phosphate bonds in lipid molecules located in the outer portion of the plasma membrane. Citrate might prevent attachment by the removal of Ca\(^{2+}\) essential for complexing with calmodulin and other cationic regulatory proteins. This thesis is quite credible now that Ca\(^{2+}\)-calmodulin has been implicated in platelet membrane receptor binding through interaction between microtubules and microfilaments. \(^{20}\) Ca\(^{2+}\)-calmodulin regulates microtubule assembly/disassembly and myosin light-chain kinase. \(^{21,22}\) Furthermore, it has been suggested that local anesthetics affect the mobility of cell surface receptors by displacing membrane bound Ca\(^{2+}\), thus altering the membrane associated microtubule–microfilament system. \(^{23}\) The complete reversal of EGTA and EDTA inhibition of attachment by Ca\(^{2+}\) alone appears to give this cation a major role in the attachment process.

Table 1. Chelator and TFP influence on unopsonized zymosan stimulation of PMN

<table>
<thead>
<tr>
<th>Chelator or TFP</th>
<th>Attachment (N)</th>
<th>Phagocytosis (N)</th>
<th>% inhibition of phagocytosis</th>
</tr>
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<tbody>
<tr>
<td>Unopsonized zymosan stimulation</td>
<td></td>
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<tr>
<td>12 mM citrate</td>
<td>1.9 ± 0.5</td>
<td>71.9 ± 4.8</td>
<td>0</td>
</tr>
<tr>
<td>1.5 mM EDTA</td>
<td>1.4 ± 0.3</td>
<td>52.0 ± 3.8</td>
<td>27.7*</td>
</tr>
<tr>
<td>4.0 mM EGTA</td>
<td>0 µM TFP</td>
<td>85.8 ± 4.1</td>
<td>8.5‡</td>
</tr>
<tr>
<td>40 µM TFP</td>
<td>0</td>
<td>65.8 ± 4.1</td>
<td>8.5‡</td>
</tr>
</tbody>
</table>

* 0.001 < P < 0.01.
† P < 0.001.
‡ Not significant.
§ P < 0.001.

Table 2. Morphometry of cytoplasmic granules

<table>
<thead>
<tr>
<th>Azurophilic granules</th>
<th>Specific granules</th>
<th>Total granules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20.4 ± 1.3</td>
<td>12.4 ± 1.0</td>
</tr>
<tr>
<td>Zymosan</td>
<td>11.5 ± 1.1</td>
<td>6.4 ± 0.8</td>
</tr>
<tr>
<td>12 mM citrate</td>
<td>21.0 ± 1.0</td>
<td>9.9 ± 0.6</td>
</tr>
<tr>
<td>1.5 mM EDTA</td>
<td>20.2 ± 1.3</td>
<td>14.0 ± 0.8</td>
</tr>
<tr>
<td>4.0 mM EGTA</td>
<td>23.3 ± 0.8</td>
<td>8.1 ± 0.4</td>
</tr>
<tr>
<td>40 µM TFP</td>
<td>22.3 ± 1.6</td>
<td>8.8 ± 0.6</td>
</tr>
</tbody>
</table>

* P < 0.001 when compared with control.
It is our hypothesis that zymosan to PMN plasma membrane attachment requires the movement of cell surface receptors in connection with the initial receptor—ligand binding. Inhibition of zymosan attachment in the cold (0–4°C) supports the view that the movement of these receptors is required for attachment. Receptor ligand binding is independent of cell metabolism and occurs at 0–4°C1,2 but when stimulation occurs by zymosan particles, the inhibition of cell surface receptor movement prevents subsequent attachment from taking place perhaps by decreased metabolism (microtubule–microfilament interaction) or membrane fluidity. Attachment of opsonized erythrocytes and opsonized *Staphylococcus aureus* to macrophage and PMN plasma membranes respectively is known to occur in the cold (0–4°C).24,25 The discrepancy between these observations and ours are unknown. However, cell type and opsonized particle differences may have led to these results.

Mg2+ is quite important to the attachment process. Ca2+ and Mg2+, used together, are most effective in reversing citrate inhibition. If the cations are used alone, Mg2+ is considerably more effective than Ca2+. Although speculative, the addition of Mg2+ to cell incubations inhibited by citrate may displace some Ca2+ from the citrate complexes and from the plasma membranes of PMN. This additional free Ca2+, together with the available Mg2+, may be sufficient to produce a greater stimulation than Ca2+ alone. In this regard Mg2+ might act, in part, through the Mg2+-ATPase of actomyosin in the microfilament system. Microfilament association with the cytoplasmic surface of the plasma membrane at the site of attachment of opsonized zymosan has been observed by Boyles and Bainton.26

Zymosan particles, once bound, are phagocytosed even in the presence of citrate. Since phagocytosis is dependent on the cytoplasmic microfilament system, a calcium-calmodulin dependent process, calcium in the outer core of endoplasm (and inner plasma membrane) may be presumed to be adequate. Alternately, Hoffstein13 has shown that membrane binding causes Ca2+ loss from the localized contact area on the plasma membrane to the cytosol. This would provide an abundance of Ca2+, activating the calcium-calmodulin system required for microfilament contraction and consequent phagocytosis, prior to the addition of citrate. It is unlikely that citrate is altering or masking the membrane receptor directly since reversal of this inhibition is complete with Ca2+ and Mg2+.

The fact that other chelators of Ca2+, such as EGTA and EDTA, inhibit zymosan attachment further strengthens the hypothesis favoring calcium involvement in attachment. In this regard it is interesting that EDTA inhibits not only attachment of zymosan but also phagocytosis of bound particles. It may be that additional Mg2+ binding by EDTA has an inhibitory effect on the cytoplasmic microfilament system, critical to the process of phagocytosis. In Figure 9, the results obtained at 12 min further elaborates this point; TFP pretreatment of PMN for 5 min severely reduces phagocytosis but reduces attachment to a lesser extent. But opsonized zymosan stimulation of PMN immediately pretreated by TFP reduces phagocytosis as a consequence of reduced attachment. TFP is known to combine with and inhibit calcium calmodulin complexes. We believe that pretreatment of PMN with TFP for 5 min may allow penetration of the drug into the cytosol and its combination with activated calmodulin. Subsequent stimulation with zymosan would produce some inhibition of attachment but primarily inhibit phagocytosis (producing a delay in the phagocytic process) and, hence, the respiratory burst. If stimulation occurs immediately after TFP treatment, then activated calmodulin in the plasma membrane will combine with the drug, inhibiting the attachment process in the membrane while having substantially less effect on the microfilament system in the cytosol, hence phagocytosis (no delay of the phagocytic process).

PMN phagocytize unopsonized zymosan only when centrifuged down to a pellet. Although the process is not understood well, it is likely that forceable, close contact of PMN and zymosan may induce zymosan-specific binding18 and consequent phagocytosis. Although citrate and EGTA (not significant) pretreated PMN inhibit this phagocytosis slightly, EDTA, TFP, and cold treatment have a dramatic inhibitory effect. High levels of inhibition of phagocytosis by the latter conditions but not the former clearly implicate Mg2+, activated calmodulin, and the microfilament system, in PMN phagocytosis by the latter conditions but not the former clearly implicate Mg2+, activated calmodulin, and the microfilament system, in PMN phagocytosis.

Ascorbate has been shown to enhance human neutrophil chemotaxis in vitro27,28 and in vivo29 without influencing their phagocytic capacity. Our studies also have failed to show any effect of this compound on PMN attachment or phagocytosis. Although we could not measure oxygen utilization or β-gluc release directly, the lack of any effect on phagocytosis is strong suggestive evidence that ascorbate does not influence the respiratory burst or enzyme release of PMN.

Our current studies help to explain the in vivo results obtained in the 45 sec, 12 mm, 4N NaOH ocular burns.3 Ascorbate has no inhibitory effect on the predominant cell type the PMN, hence its failure to prevent or reduce corneal ulcers in this burn might be expected. The favorable effect of citrate in this experiment is probably based on inhibition of the PMN. Although we mea-
sured the reduction in β-gluc release from PMN (contained in azurophilic granules) in the presence of citrate, it is likely that this applies to all enzymes elaborated by the cell including collagenase, elastase, cathepsins, and acid hydrolases. Our electron microscopic studies, showing the inhibition of the release of specific and azurophilic granules containing these enzymes, strongly support this contention.

Edetic acid has been studied in vivo and in vitro for its putative inhibitory effects on collagenase in the cornea. Na-EDTA, and CaNa2EDTA inhibit cell free crude preparations of collagenase by virtue of their Ca2+ or Zn2+ chelating properties. The results of our own studies on inhibition of the PMN by EDTA and other chelating compounds suggests a two pronged effect for in vivo studies. The first is by prevention of collagenase release from the PMN source and second by inhibiting the activity of the small amount of collagenase that might be released.

PMN enter the burned cornea under the influence of an unknown chemotactic agent. Once in the cornea, PMN probably elaborate chemotactic substances that call in additional PMN, thus creating a sterile infiltrate. One byproduct of PMN respiration, superoxide radicals, not only damage tissues but react with lipids and human serum albumin to form active complexes chemotactic in nature. Inhibition of PMN respiration by extracellular cation chelation would reduce or eliminate the source of these radicals. The influence of citrate on PMN locomotion and the production of chemotactic substances is the subject of our current research.

The topical use of a naturally occurring nontoxic substance (ie, citrate) to inhibit PMN stimulation is an especially attractive approach to the prevention of ulceration in the alkali burned eye. This approach also opens avenues for the treatment of a host of other eye diseases marked by PMN stimulation. In fact, it would be foolish to ignore the possibility that generalized systemic diseases, induced in part by PMN stimulation, might be favorably influenced by supplementation with large amounts of citrate.

Key words: alkali, cornea, polymorphonuclear leukocytes, citrate, calcium

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References


