The Effect of Sodium Citrate on the Stimulation of Polymorphonuclear Leukocytes

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Topical administration of sodium citrate reduces the incidence of corneal ulceration and perforation following an alkali burn to the eye. The specific mechanism by which sodium citrate prevents the ulceration is not understood, although citrate does inhibit the infiltration of polymorphonuclear leukocytes (PMNs) into the cornea following an alkali burn. In the present study, the effects of sodium citrate and another calcium chelator, ethylene glycol bis (β-aminoethylether)-N,N'tetraacetic acid (EGTA), upon PMN oxygen consumption and lysosomal enzyme release were determined. Oxygen consumption was measured polarographically using a Clark-type oxygen electrode, and lysosomal enzyme release was determined by intra- and extra-cellular measurements of myeloperoxidase activity. Opsonized zymosan and N-formylmethionylleucylphenylalanine (FMLP) were used to stimulate neutrophil oxygen consumption and lysosomal release. Both sodium citrate and EGTA inhibited PMN oxygen consumption and lysosomal enzyme release in response to opsonized zymosan. In contrast, neither sodium citrate nor EGTA reduced PMN oxygen consumption or lysosomal enzyme release in response to FMLP. Therefore, the ability of sodium citrate (and EGTA) to inhibit PMN stimulation is dependent upon the choice of stimulus. Until the inflammatory mediators involved in the ulcerative process following an alkali burn to the eye are delineated, the impact of sodium citrate upon PMN stimulation in vivo cannot be resolved. Invest Ophthalmol Vis Sci 26:1257–1261, 1985

Severe alkali burns on the surface of the eye produce a variety of pathologic changes culminating in corneal ulceration and perforation. Topical administration of sodium citrate has been found to reduce the incidence of corneal ulceration and perforation usually observed in rabbits following a chemical injury of this type.[1,2] While the precise mechanism by which it reduces the incidence of corneal ulceration is unknown, sodium citrate has been shown to inhibit the infiltration of polymorphonuclear leukocytes (PMNs) into the corneas following an alkali burn of the rabbit eye.[3] Since PMNs are believed to play a central role in the pathogenesis of corneal ulceration,[4-6] it is possible that reducing the infiltration of PMNs into the cornea decreases the incidence of ulceration. Sodium citrate has also been shown to reduce PMN infiltration in ocular inflammation induced by endotoxin.[7]

The role of citrate in many biochemical pathways and its powerful divalent cation chelating properties allow for numerous possible avenues by which this compound could interfere with the corneal ulceration process following an alkali burn to the eye. However, Pfister et al[8] have recently demonstrated that sodium citrate inhibits the stimulation of, and subsequent release of enzymes from PMNs in response to opsonized zymosan, an effect attributed to the chelation of extracellular calcium. This inhibitory effect of sodium citrate on the stimulation of PMNs would, therefore, seem to afford a plausible explanation for citrate therapy abrogating the progression of ulceration in the alkali burned cornea. Unfortunately, the actual inflammatory mediators responsible for stimulating PMNs in the alkali burned cornea have not been identified, and it is not possible to state with certainty that citrate inhibits PMN stimulation in this in vivo situation. Yet, we are aware that leukocytes can be stimulated by different mechanisms: one activated typically by opsonized zymosan, the other by a variety of peptides such as FMLP.[9-11] It seems reasonable to assume, therefore, that the inflammatory mediators generated in the alkali burned cornea would stimulate infiltrating PMNs by one (or both) of these mechanisms.

In this study, we have compared the influence of sodium citrate on PMN stimulation by opsonized

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zymosan or FMLP. In addition, we have examined the effect of another calcium chelating agent, EGTA, upon leukocyte stimulation.

Materials and Methods

Materials

Cytochalasin B, N-formylmethionylleucylphenylalanine (FMLP), zymosan, hexadecyltrimethylammonium bromide (HTAB) and dimethylsulphoxide (DMSO) were purchased from Sigma Chemical Company (St. Louis, MO). Sodium citrate and ethylene glycol bis(λβ-aminoethylether)N,N'-tetraacetic acid (EGTA) were purchased from Baker Chemical Company (Phillipsburg, NJ). Other materials were reagent grade.

Hank's balanced salt solution (HBSS) was prepared daily from stock solutions as described previously.[9] Stock solutions of cytochalasin B (50 μg/ml in 0.5% (v/v) DMSO:HBSS) and FMLP (10^{-4} M in 2.5% (v/v) DMSO:HBSS) were stored frozen in separate aliquots and thawed immediately before use.

Cell Preparation

Human leukocytes were prepared from the peripheral blood of normal healthy volunteers as previously described elsewhere.[12] Informed consent was obtained after the nature of the procedure to draw blood had been explained fully. In brief, methyl cellulose sedimentation was used to obtain PMNs from whole blood; contaminating erythrocytes were lysed in an 0.82% solution of ammonium chloride in hypotonic potassium chloride solution. The final leukocyte suspension contained 85% neutrophils with the remainder being lymphocytes and eosinophils.

Addition of Stimulating Agents and Calcium Chelators

Zymosan was opsonized immediately prior to each experiment. Zymosan (20 mg/ml) was incubated in fresh human serum for 20 min at 37°C, then washed and resuspended to give a final concentration of 2 mg/ml.

Stimulation of PMNs with FMLP is augmented in the presence of cytochalasin B.[13] Therefore, cytochalasin B was added to the PMNs (final concentration, 5 μg/ml) 15 min prior to the addition of the stimulating agent, FMLP (final concentration, 10^{-5} M). Control experiments were performed where PMNs were incubated in the presence of cytochalasin B alone.

Stock solutions of sodium citrate and EGTA were made fresh daily in HBSS; the pH was adjusted to 7.4 with hydrochloric acid (0.1 N) or sodium hydroxide (1 N), respectively. Sodium citrate or EGTA were added to the cell suspension 5 min (enzyme release) or 10 min (oxygen consumption) prior to the addition of opsonized zymosan or FMLP.

Oxygen Consumption

Oxygen consumption was measured polarographically using a Clark-type oxygen electrode (Yellow Springs, CA) at 37°C; 1.7 ml of cell suspension (3–6 × 10^6 cells/min) in HBSS was allowed to equilibrate for 10 min in the chamber in the presence or absence of EGTA or sodium citrate before addition of the prewarmed (37°C) stimulating agent. The dissolved oxygen concentration was monitored continuously for 5 min prior to and 10 min after addition of the stimulus. Control experiments were performed in the absence of PMNs.

Lysosomal Enzyme Release

Cell suspensions (5 × 10^6 cells/ml) were allowed to equilibrate in a water bath (37°C) for 30 min. The incubation period following the addition of FMLP was 15 min and for opsonized zymosan-treated cells, the incubation period was 30 min. These time periods were found to be optimal. PMN degranulation was assessed by measuring myeloperoxidase release. The data were expressed as the percentage of total cellular enzyme which was released into the supernatant. In each experiment, spontaneous degranulation was determined for neutrophils not exposed to any stimulus; this percentage was subtracted from the experimental (stimulated) levels. Myeloperoxidase activities were determined as follows: At the completion of incubation of leukocytes with FMLP or opsonized zymosan, the PMNs were placed on ice and then centrifuged to obtain a clear supernatant and cell pellet. Myeloperoxidase activity in the supernatant was determined by adding an equal volume of 0.5% HTAB, freeze-thawing three times, and assaying it as previously described.[14] Intracellular myeloperoxidase activity was determined by adding 1 ml of HTAB (0.5%) to the pellet, mixing thoroughly, freeze-thawing three times, and assaying it as above.[14]

Leukocyte viability in the presence or absence of FMLP, opsonized zymosan, sodium citrate or EGTA was determined by measuring lactate dehydrogenase (LDH) activity. LDH activity in the supernatant and cell pellet was assayed using the method of Cabaud and Wroblewski[15] following lysis of the cells in 0.2% Triton X-100 at 37°C for 30 min.

Free Calcium Ion Concentrations

The free calcium ion concentration (calcium activity) in HBSS in the presence or absence of citrate or

Table 1. The release of azurophillic myeloperoxidase (MPO) by PMNs in response to a variety of stimuli

		Units MPO/5 × 10^6 PMNs	
Stimulus	*n*	*Intra-cellular*	*Extra-cellular*
None	3	559 ± 57	14 ± 3
Opsonized zymosan	3	507 ± 65	37 ± 8
Cytochalasin B	5	658 ± 212	14 ± 2
FMLP + Cytochalsin B	5	480 ± 163	176 ± 39

Each value is the mean ± SEM for experiments on purified PMNs obtained from (n) donors.

EGTA was determined using a calibrated calcium-sensitive electrode (Orion, series 93, Orion Research, Inc.; Cambridge, MA).

Results

The effects of opsonized zymosan and FMLP on the release of myeloperoxidase by PMN's are shown in Table 1. FMLP (in the presence of cytochalasin B) and opsonized zymosan stimulated the release of myeloperoxidase from within the leukocytes, the percentage of released enzyme being in close agreement with values reported by others.[16,17] Addition of cytochalasin B, alone, had no effect on the enzyme release or oxygen consumption of resting PMNs.

Sodium citrate (15 mM) and EGTA (3.5 mM) markedly inhibited the increased oxygen consumption and lysosomal enzyme release by PMN preparations stimulated by opsonized zymosan (Fig. 1). In contrast, when FMLP was used to stimulate PMN oxygen consumption and lysosomal enzyme release, no significant inhibition was observed in the presence of sodium citrate or EGTA (Fig. 2).

Examination of the patterns of oxygen consumption by PMNs following exposure to the two stimuli show clearly that sodium citrate and EGTA inhibited the cellular response to opsonized zymosan. However, it is also quite evident that both sodium citrate and EGTA were ineffective in reducing the response to FMLP (Fig. 3). It is noteworthy that even when the effect of opsonized zymosan on PMN oxygen consumption was completely abolished by a high concentration of sodium citrate (30 mM), stimulation of that same cell suspension with FMLP was unaffected (Fig. 4).

Measurement of LDH levels in the supernatant and cell pellet revealed LDH release to be less than 3% with each experimental treatment. Thus, cellular integrity was not affected by citrate, EGTA, FMLP or opsonized zymosan.

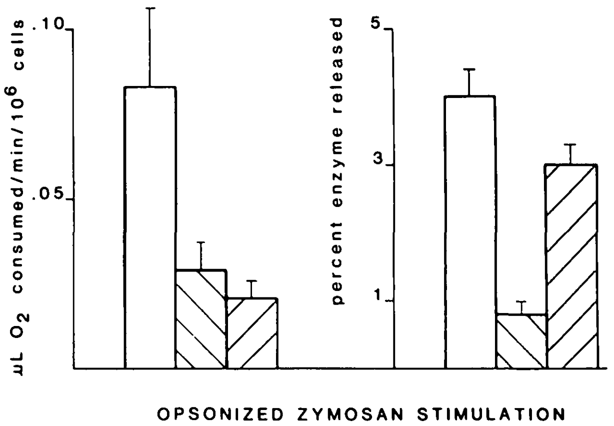

Fig. 1. The effect of sodium citrate and EGTA on the stimulation of PMNs by opsonized zymosan. The stimulated oxygen consumption and enzyme release in response to opsonized zymosan are markedly inhibited in the presence of 15 mM sodium citrate ▧ or 3.5 mM EGTA ▨. Each value is the mean ± SEM for at least three separate experiments.

The addition of sodium citrate (final concentration 15 mM) to HBSS reduced the "free" calcium concentration from 1.3 × 10^{-3} M to 2.5 × 10^{-5} M, while EGTA (final concentration, 3.5 mM) exhibited greater chelation activity, reducing the "free" calcium to approximately 3.7 × 10^{-7} M.

Discussion

In this study, we have confirmed that sodium citrate and EGTA, at concentrations that chelate calcium, inhibit PMN stimulation in response to opsonized zymosan.[8] However, we have also reported

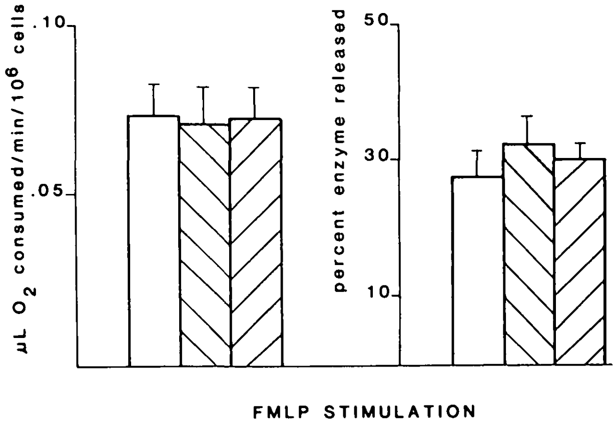

Fig. 2. The effect of sodium citrate and EGTA on the stimulation of PMNs by FMLP. The stimulated oxygen consumption and enzyme release in response to FMLP are unaffected in the presence of 15 mM sodium citrate (▧) or 3.5 mM EGTA (▨). Each value is the mean ± SEM for at least four separate experiments.

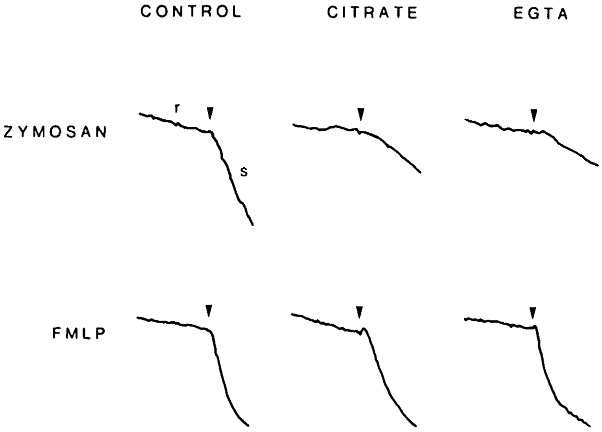

Fig. 3. Oxygen consumption of PMNs in the presence or absence of sodium citrate or EGTA. After recording the oxygen consumption of the resting PMNs (r), FMLP or opsonized zymosan was added (▼); the steep decrease in oxygen concentration indicates stimulation (s) of cells. Sodium citrate (15 mM) and EGTA (3.5 mM) abrogated the response of the PMNs to opsonized zymosan but did not affect the response to FMLP. Vertical bar: 20 μl O_2; horizontal bar: 2 min.

a previously unpublished finding that the stimulation of PMNs in response to FMLP is not changed in the presence of either citrate or EGTA.

These findings support the concept that extracellular calcium is required to facilitate the stimulation of PMNs with opsonized zymosan.[8,16] Therefore, it is not surprising that citrate and EGTA, both powerful calcium chelating agents, prevent the stimulation of PMNs by opsonized zymosan. Indeed, this inhibitory effect of citrate or EGTA on PMN stimulation can be reversed by the addition of calcium to the medium.[8]

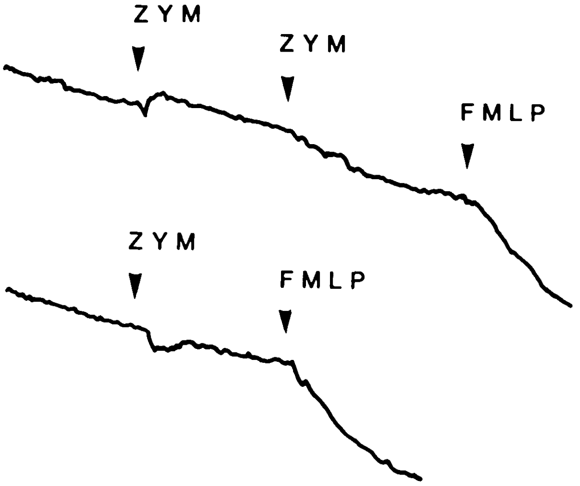

Fig. 4. Oxygen consumption of PMNs in the presence of a high concentration of sodium citrate. PMNs were suspended in HBSS containing 30 mM sodium citrate and the resting oxygen consumption recorded. The top trace shows that challenge with opsonized zymosan (X2) resulted in no change in oxygen consumption, whereas addition of FMLP still produced stimulation of the leukocytes. The possibility that the response to FMLP occurred as a consequence of the cells being in an excessive concentration of stimulants is excluded by examination of the lower trace of a similar experiment. Here the response to opsonized zymosan (X1) was completely abolished in the presence of sodium citrate but again the response to FMLP was unaffected. Vertical bar: 20 μl O_2; horizontal bar: 2 min.

The dependency of PMN stimulation by FMLP upon extracellular calcium has not been so clearly established. However, a recent study showed that FMLP-induced changes in intracellular calcium, which are critical for neutrophil stimulation, are insensitive to extracellular EGTA.[18] In contrast, depletion of intracellular calcium effectively inhibits stimulation of PMNs by FMLP.[19] Thus, in the present study, citrate was only added to the medium 5–10 min before the stimulating agent to minimize the effect of this cation chelating agent on intracellular calcium. In any event, there seems little doubt that FMLP and opsonized zymosan stimulate leukocytes by different mechanisms.[11] Presumably, the inflammatory mediators generated in the alkali burned cornea could stimulate leukocytes by one or both of these mechanisms. Consequently, the impact of citrate upon PMN stimulation in the alkali burned eye will not be resolved until the inflammatory mediators involved in the ulcerative process have been delineated.

Since it has already been established that sodium citrate reduces PMN infiltration into the alkali-burned cornea,[3] the possibility that citrate may cause inhibition of PMN stimulation in vivo may be relatively less important than the other mechanisms by which this compound could affect the inflammatory process. For example, by chelating divalent cations, sodium citrate could interfere with PMN locomotion, with complement activation, and also decreased production of chemotactic factors. Extracellular calcium and magnesium are required for chemolocomotion measured with a Boyden chamber,[20] but whether this is true of in vivo locomotion is not known. Complement activation is both calcium- and magnesium-requiring,[21,22] and cation chelation could decrease complement activation and the production of chemotactic factors. The generation of inflammatory mediators via arachidonic acid metabolism could also be adversely affected by cation chelation, but the necessity for calcium or magnesium has not yet been defined for these pathways. Therefore, future work better characterizing the basic pathologic processes which follow an alkali burn to the eye will help to explain the mechanism of action of sodium citrate.

Key words: sodium citrate, EGTA, cation chelation, calcium, polymorphonuclear leukocyte, cornea, inflammation

Acknowledgments

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