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Divalent Cation Requirements for Leukocyte Adhesiveness* (33701)

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Both polymorphonuclear leukocyte (PMN) aggregation and PMN adhesion to glass are increased after phagocytosis of bacteria or exposure to bacterial endotoxin (1). The PMN adhesiveness is abolished by chelation with EDTA and is considered to be a divalent cation-dependent process (2, 3). A significant disadvantage of chelation studies with EDTA is that this agent binds calcium and magnesium with approximately the same avidity and, therefore, cannot be used to differentiate the effects of calcium and magnesium ion on cell function. Moreover, the magnesium salt of EDTA cannot be used to differentiate calcium ion and magnesium ion effects because plasma containing magnesium EDTA has appreciable concentrations of calcium ion (4). In contrast, the magnesium salt of ethylene glycol diamino ethyl tetraacetic acid (EGTA) is an effective agent for distinguishing between Mg^{2+} and Ca^{2+} requirements since EGTA binds calcium ion 100,000-fold more avidly than magnesium ion (4). Plasma containing MgEGTA has a slight excess of magnesium ion and is virtually free of calcium ion.

In the present study the effects of the sodium and the magnesium salts of EDTA and EGTA on PMN adhesion to glass and on human PMN aggregation after exposure to endotoxin were determined.

Methods. Blood from normal human donors was heparinized with 4 mg/100 ml of preservative-free heparin.[2] Chelating agents were added to blood to achieve 10 m*M* concentration of various salts of EDTA[3] or EGTA.[4] Equal volumes of saline were added to control specimens.

Techniques for determination of leukocyte adhesion were previously described (5). In brief, leukocytes in horizontally placed capillary tubes were allowed to sediment to the side for 1 hr at 37°. The tubes were then centrifuged at 16,000 rpm for 10 sec and the number of leukocytes remaining adherent to

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TABLE I. Effect of Chelating Agents on Leukocyte Adhesion to Glass.

Additives	Calcium ion conc[a] (M)	Magnesium ion conc[a] (M)	Adhesion index[b]
1. Saline	2.5×10^{-3}	1.0×10^{-3}	204
2. Na_2H_2EDTA	9.7×10^{-12}	3.1×10^{-10}	0
3. Na_2H_2EGTA	3.8×10^{-12}	9.7×10^{-7}	0
4. MgEDTA	1.5×10^{-5}	3.5×10^{-3}	503
5. MgEGTA	7.2×10^{-9}	3.5×10^{-3}	460

[a] Calculations based on the following assumptions: $[Ca] = 2.5 \times 10^{-3} M$; $[Mg] = 1.0 \times 10^{-3} M$; [EDTA] or [EGTA] $= 10 \times 10^{-3} M$. The effects of ionic strength, pH, protein binding, and other divalent cations were assumed to be negligible.

[b] Number of cells adherent to capillary tube walls (mean of 10 tubes).

capillary tube walls were counted. Ten capillary tubes were used for each variable tested.

The effect of chelating agents on endotoxin-induced PMN aggregation was determined as follows: Blood smears were made from heparinized whole blood which had been rotated in siliconized test tubes for 15 min with 30 μg/ml of bacterial endotoxin (*E. coli*, Difco 0127:B8). The indicated chelating agent or saline was then added, and specimens were rotated for a second 15-min period. Blood smears were made after the second rotation, and stained with Wright's stain. The percentage of 100 PMNs present in aggregates before and after addition of chelating agents was determined.

Results. As demonstrated in Table I, divalent cation depletion by Na_2H_2EDTA or Na_2H_2EGTA virtually abolished leukocyte adherence to glass whereas the magnesium salt of both EDTA and EGTA resulted in increased leukocyte adherence. The effect of chelating agents on leukocyte aggregation induced by bacterial endotoxin is shown in Table II. The sodium salts of EGTA and EDTA produce disaggregation of previously aggregated leukocytes. In contrast, magnesium salts of both EDTA and EGTA produced little change.

Discussion. Specific divalent cation requirements for various PMN functions have been technically difficult to evaluate. Deionization of plasma with resins does not lower cation concentrations as effectively as available chelating agents and, once plasma has been treated with resins, specific cation concentrations cannot be accurately quantitated. Na_2H_2EDTA is capable of providing very low plasma concentration of free calcium and magnesium ions. However, as pointed out by Hovig, this agent binds calcium and magnesium with approximately the same avidity (4). In the presence of a slight excess of Na_2H_2EDTA, the calcium and magnesium concentrations are reduced to approximately the reciprocal of their respective association constants with the common ligand; $(Mg^{2+}) = 10^{-8.7} M$, $(Ca^{2+}) = 10^{-10.6} M$ (6). When the sum of the concentrations of calcium ion and magnesium ion exceeds the concentration of the common ligand, as is the case when 10 mM MgEDTA is added to blood, magnesium ion is displaced by calcium ion in proportion to the ratio of the association constants of EDTA for magnesium and calcium. Under these circumstances unbound calcium concentration is approximately one-hundredth the unbound magnesium ion concentration (4) (Table I).

The inability of MgEDTA to produce profound hypocalcemia is demonstrated by the failure of MgEDTA to inhibit complement-dependent hemolysis, a process known to re-

TABLE II. Effect of Chelating Agents on PMN Aggregation Produced by Prior Rotation with Endotoxin.

Additives after rotation with 30 μg/ml of endotoxin	PMN present in aggregates[a] (%)
1. None[b]	30
2. Saline	36
3. Na_2H_2EDTA	3[c]
4. Na_2H_2EGTA	7[c]
5. MgEDTA	33
6. MgEGTA	25

[a] Mean of 100 PMN counted in each of five experiments.

[b] Percentage of cells in aggregates after initial rotation with endotoxin. All others were counted after a second 15-min rotation with additives as indicated.

[c] ($p < 0.001$) by Student's t test.

quire ionized calcium (7). In contrast, the magnesium salt of EGTA lowers plasma calcium concentration approximately 10,000-fold lower than MgEDTA and does inhibit complement-dependent hemolysis of sensitized sheep erythrocytes (7). By using MgEGTA it was possible to determine that leukocyte adhesion to glass or to other leukocytes was not inhibited by calcium ion concentrations of less than 10^{-8} *M*.

Previous studies have not determined specific calcium ion requirements for leukocyte adhesion. Both Brittingham and Garvin observed that PMN adhesion was inhibited by EDTA (2, 3). Similarly, Garvin observed that deionization of plasma with resins inhibited leukocyte adhesiveness which could not be restored by addition of calcium alone to treated plasma. Addition of magnesium alone restored adhesiveness but both calcium and magnesium ions were required for optimal adhesiveness. Allison and co-workers observed that divalent cation depletion by resins or EDTA impaired the capacity of neutrophils to phagocytize bacteria and to stick to glass (8). Addition of magnesium ion or calcium ion in quantities sufficient to saturate EDTA binding sites restored phagocytic and adhesive properties. These authors also concluded that magnesium ions were more effective than calcium in restoring adhesiveness but that both were required for optimal cell adherence. Similarly, while studying the effect of EDTA on leukocyte adherence to glass, Rabinowitz concluded both calcium and magnesium were necessary for effective adherence (9). The present findings differ from previous studies in only two respects. Using a chelating agent (MgEGTA) which produces profound hypocalcemia in the presence of a slight excess of magnesium it is possible to demonstrate (a) that PMN adhesion is not specifically a calcium-dependent process, and (b) that magnesium ion can actually increase adhesiveness of PMNs in the virtual absence of ionized calcium. That leukocyte adherence to glass or to other leukocytes is not a complement-dependent process is suggested by the fact that complement-dependent hemolysis is calcium-ion dependent and leukocyte adherence is not.

Summary. Using a chelating agent which binds calcium ions 100,000-fold more avidly than magnesium ions (EGTA) it was demonstrated that PMN adhesion to glass or to other leukocytes was not a calcium–dependent process and that a slight excess of magnesium ion actually caused increased PMN adhesiveness in the virtual absence of calcium.

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