Substances Which Aggregate Neutrophils

Mechanism of Action

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Several agents which influence calcium fluxes in neutrophils were tested for their influence on human neutrophil aggregation. Formyl-methionyl-leucyl-phenylalanine, a synthetic chemotactic tripeptide, aggregated the cells. Cytochalasin B and high levels of extracellular calcium or phosphate enhanced this effect; $10^{-4}$ M to $10^{-4}$ M lanthanum inhibited it. In addition, the calcium ionophore A23187 aggregated the cells. Aggregation induced by the chemotactic factor and A23187 required extracellular calcium. These results correlate with the known or postulated ability of chemotactic factors, A23187, calcium, phosphate, lanthanum, and cytochalasin B to enhance or inhibit the influx and intracellular accumulation of the calcium ion. Transmembrane fluxes or intracellular levels of calcium may modulate PMN aggregation. Aggregation induced by the chemotactic tripeptide and A23187 also required extracellular magnesium. Since calcium and magnesium cannot substitute for each other in the aggregation response to the chemotactic factor or A23187, each bivalent cation must play a separate role in PMN aggregation. The role of magnesium is unknown. Since magnesium, unlike calcium, is known to be necessary for PMN adherence to glass, it may play a permissive role in PMN aggregation. Thus, magnesium may foster the formation of cell-cell adhesions. In addition to inhibiting chemotactic factor-induced aggregation at concentrations of $10^{-4}$ M to $10^{-4}$ M, lanthanum, at concentrations of $10^{-4}$ M to $10^{-4}$ M, aggregated the cells. Lanthanum-induced aggregation did not require extracellular calcium or magnesium. This aggregation may result from the formation of intercellular adhesions by the lanthanum ion directly. (Am J Pathol 92:155-166, 1978)

CHEMOTACTIC FACTORS stimulate various polymorphonuclear neutrophil (PMN) responses such as directed migration and degranulation. In evoking these responses, chemoattractants bind to the cell surface and, it has been postulated, induce the formation or the accumulation of an intracellular mediator which initiates or modulates various biochemical sequences involved in cellular and organelar motility. Ionized calcium may be such a mediator. Thus, the PMN can be viewed as a secretory or contracting cell, and Ca$^{2+}$ can be considered a coupler of membrane excitation to actinmyosin contraction, microtubule assembly, cyclic nucleotide forma-
tion, or organelle-surface–membrane fusion. These postulates have been strengthened by the finding that chemotactic factors enhance the transmembrane fluxes and exchangeable intracellular pool of Ca\(^{2+}\).

Recently, chemotactic factors have been shown to stimulate the aggregation of rabbit peritoneal and human blood PMNs. In this report we examine the influence of factors which modulate intracellular Ca\(^{2+}\) levels on human PMN aggregation.

**Materials and Methods**

**Chemotactic Factor**

The synthetic chemotactic tripeptide formyl–methionyl–leucyl–phenylalanine (FMLP) was obtained and used as previously described.

**Reagents**

Cytochalasin B (Aldrich Chemical Company, Milwaukee, Wis.) and FMLP were dissolved in dimethylsulfoxide. In the final concentrations used in this study (0.02% or less), the solvent did not influence PMN function. The bivalent cation ionophore A23187 was a generous gift of Dr. Robert Hamill of the Eli Lilly Company, Indianapolis, Indiana. The buffer was a modified Hanks’ balanced salt solution containing (mM): NaCl, 130; KCl, 5.5; Na\(_2\)HPO\(_4\), 0.6; NaH\(_2\)PO\(_4\), 0.6; glucose, 10; and tris, 25. For some experiments the phosphate salts were omitted from the buffer. Where indicated, La\(^{3+}\), Mg\(^{2+}\), or Ca\(^{2+}\) were added to the buffers in the form of chloride salts. Chemicals were of reagent grade or better, and buffers were adjusted to pH 7.4 before use.

**Neutrophils**

Normal human whole blood was centrifuged over Ficoll–Hypaque discontinuous gradients to obtain leukocyte populations containing greater than 96% PMNs.

**Aggregation**

PMNs were freed from contaminating erythrocytes by hypotonic lysis and then washed and suspended (4600 PMN/\(\mu\)l) in the appropriate buffer. One milliliter of the suspension was placed in a plastic vial and stirred continuously with a magnetic bar. For each experiment, an aggregating substance was added directly to the cell suspension and 25-\(\mu\)l samples were taken at 1/4, 1/2, 1, 2, 4, 8, and 15 minutes thereafter. Samples were immediately diluted in 10 ml of Isoton solution (Coulter Electronics, Hialeah, Fla.) and analyzed with a Coulter Counter, Model ZBI, equipped with a Volume Channelizer II. For each sample, the counter was set to enumerate the concentration of particles greater than 60 fl (called T) and greater than 520 fl (called A). Since human PMNs are approximately 330 fl, T is the total particle concentration and A is the large or aggregated particle concentration. When PMNs aggregate, T falls while A rises. To quantitate these changes, the percentage of large particles (100 \times A/T) for each sample and the aggregation index (the mean of the percentage of large particles found 1/4 and 1 minute after the addition of the aggregating substance minus the pre-addition percentage of large particles) for each experiment was calculated.
Enzyme Release

At 2 and 15 minutes after adding an aggregating substance to the PMN suspension, ½-
ml samples were taken, chilled in ice, and centrifuged at 4 C; their supernatant fluids were
analyzed for lysozyme, β-glucuronidase, and lactate dehydrogenase (LDH), as previously
described.4,18

Results

Influence of Cytochalasin B on PMN Aggregation

FMLP, as previously described,30 aggregated human PMNs. This effect
was reflected in an increase in the percentage of large particles formed
after adding 5×10⁻⁶ M FMLP to the suspension of PMNs (Text-figure 1, solid line). The magnitude of these changes was proportional to the
amount of FMLP added to the suspension over a range of 10⁻⁸ M to
5×10⁻⁴ M (not shown). Cytochalasin B, by itself, did not aggregate the
cells (Text-figure 1, dotted line) but did enhance FMLP-induced aggrega-
tion strikingly (Text-figure 1, dashed line). Concentrations of cytochalasin
B from 0.1 to 5.0 μg/ml enhanced FMLP-induced aggregation (not
shown). These results are similar to those found for rabbit peritoneal
PMNs.29

Influence of A23187 on PMN Aggregation

In concentrations of 10⁻⁷ M and 10⁻⁶ M the bivalent cation ionophore
A23187 aggregated the PMNs (Text-figure 2). Unlike chemotactic-factor-
induced aggregation which abated within 1 to 4 minutes and had a

Text-figure 1—Large particle percentage of PMN suspensions treated with cytochalasin B and/or
FMLP. The buffer contained 1.4 mM Ca²⁺, 0.7 mM Mg²⁺, and 1.2 mM PO₄³⁻.
steadily increasing, monophasic dose–response curve, A23187-induced aggregation progressively increased over 15 minutes and had a biphasic dose–response curve: $10^{-5}$ M A23187 induced much less aggregation than did $10^{-6}$ M (Text-figure 2). The cause for this biphasic dose–response curve is unknown. However, cells exposed to $10^{-5}$ M A23187 showed an 18.5% decrease in mean cell volume, rapidly degranulated (Table 1), and did not aggregate in response to FMLP (not shown). Therefore, high

Table 1—Enzyme Release in Neutrophils Exposed to Aggregating Substances for 2 or 15 minutes

| Aggregating substance | Lysozyme  | β-Glucuronidase | Lactate dehydrogenase |
|-----------------------|-----------|-----------------|-----------------------|
| None                  | 3.6 ± 2.2* 6.0 ± 2.0 | 3.3 ± 1.3 5.5 ± 2.0 | 5.8 ± 3.7 10.3 ± 0.8 |
| FMLP (5 × 10⁻⁴ M)     | 4.8†       | 5.3             | 3.7                   | 7.1         |
| La⁺⁺ (1 × 10⁻⁴ M)     | 2.2       | 6.8             | 2.6                   | 4.5         |
| La⁺⁺ (1 × 10⁻³ M)     | 2.1       | 4.5             | 2.2                   | 4.3         |
| FMLP (5 × 10⁻⁴ M)     | 2.2       | 6.8             | 2.6                   | 4.5         |
| A23187 (1 × 10⁻⁴ M)   | 61.0      | 73.1            | 33.1                  | 60.6        |
| A23187 (1 × 10⁻³ M)   | 13.9      | 35.9            | 7.9                   | 18.9        |
| A23187 (1 × 10⁻⁵ M)   | 6.4       | 21.4            | 4.1                   | 5.4         |
| A23187 (1 × 10⁻⁶ M)   | 4.0       | 8.4             | 3.4                   | 4.5         |
| Cytochalasin B (5 μg/ml) | 4.1       | 6.8             | 3.7                   | 4.1         |
| Cytochalasin B (5 μg/ml) + FMLP (5 × 10⁻⁴ M) | 42.0 | 49.0 | 38.0 | 39.8 |

* Mean ± SD for four experiments, as percentage of total cellular enzyme
† Mean of two experiments, as percentage of total cellular enzyme
concentrations of A23187 may interfere with cell–cell adhesiveness either directly or by contracting or degranulating the cells.

Influence of La$^{3+}$ on PMN Aggregation

La$^{3+}$ easily precipitates with multivalent anions. Therefore, in these studies a buffer which omitted phosphates was used. In concentrations from $10^{-5}$ M to $10^{-3}$ M, La$^{3+}$ aggregated the PMNs (Text-figure 3). At $5 \times 10^{-4}$ M and $10^{-3}$ M aggregation was intense (aggregation index, 47.4 ± 3.6 and 44.9 ± 1.7 SEM, respectively) and remained prominent even 15 minutes after the addition. At concentrations of $10^{-4}$ M and below, La$^{3+}$ did not aggregate the cells (Text-figure 3). At $10^{-6}$ M and $10^{-5}$ M, La$^{3+}$ inhibited FMLP-induced aggregation (Text-figure 4). At $10^{-5}$ M, La$^{3+}$ induced slight aggregation (aggregation index, 2.8 ± 0.9 SEM); FMLP, when added to these suspensions, did not aggregate (aggregation index, 2.2 ± 0.4 SEM) the cells above this slight background. Thus, at $10^{-5}$ M, La$^{3+}$ nearly totally inhibited aggregation induced by FMLP.

Influence of Phosphates on PMN Aggregation

FMLP-induced aggregation of PMNs suspended in the medium free of phosphates was significantly less than that found for PMNs suspended in the medium containing phosphates (aggregation index, 4.8 ± 0.4 and 9.9 ± 2.0 SEM, respectively, $P < 0.005$). Adding increasing amounts of phosphate (as pH 7.4 phosphate buffer) to PMN suspensions resulted in increasing enhancement of FMLP-induced aggregation (Text-figure 5).

![Text-Figure 3](image_url)

**Text-Figure 3**—Large particle percentage of PMN suspension treated with varying amounts of lanthanum chloride. The buffer contained 1.4 mM Ca$^{2+}$, 0.7 mM Mg$^{2+}$, and no PO$_4^{3-}$. 
Influence of Ca\(^{2+}\) and Mg\(^{2+}\) on PMN Aggregation

Both Ca\(^{2+}\) and Mg\(^{2+}\) were required for A23187- and FMLP-induced aggregation (Text-figures 6 and 7); neither bivalent cation was required for La\(^{3+}\)-induced aggregation (Text-figure 7). Increases in Ca\(^{2+}\) with (Text-figure 6, upper panel) or without (Text-figure 6, center panel) equivalent increases in Mg\(^{2+}\) led to progressive increases in the magnitude of FMLP-induced aggregation. Increases in Mg\(^{2+}\) above 0.7 mM were not associated with these changes when Ca\(^{2+}\) was held constant at 1.4 mM (Text-figure 6, lower panel).

Influence of Aggregating Substances on Enzyme Release

The cytosolic enzyme LDH was not released from the PMN under any of the conditions studied (Table 1). Two aggregating substances, A23187 and FMLP (with cytochalasin-B–treated cells), induced prominent release of the granule-bound enzymes, β-glucuronidase and lysozyme, whereas two other aggregating substances, La\(^{3+}\) and FMLP (with PMNs not exposed to cytochalasin B), did not (Table 1). These results suggest that PMNs remain viable during the aggregation experiments. Apparently, aggregation can occur in the absence of prominent degranulation.

Discussion

Chemotactic factors and A23187 induce an influx of extracellular Ca\(^{2+}\) and an increase in the exchangeable pool of Ca\(^{2+}\) in the PMN.\(^ {13,16,17,19,25,26}\) High levels of extracellular Ca\(^{2+}\),\(^ {25,26}\) cytochalasin B,\(^ {26}\) and possibly extra-
cellular phosphates\textsuperscript{9} enhance these effects whereas La\textsuperscript{3+} inhibits them.\textsuperscript{16,19} Our studies suggest that Ca\textsuperscript{2+} influxes and the attendant increases in cell-associated Ca\textsuperscript{2+} trigger aggregation. Thus, the chemotactic tripeptide FMLP aggregates PMNs, and this effect is enhanced by high levels of extracellular Ca\textsuperscript{2+} (Text-figure 6, upper and center panels), cytochalasin B (Text-figure 1), and extracellular phosphates (Text-figure 5). A23187 also aggregates the cells (Text-figure 2). At 10\textsuperscript{-6} M and 10\textsuperscript{-5} M, La\textsuperscript{3+} inhibits FMLP-induced aggregation (Text-figure 4). Finally, FMLP- and A23187-induced aggregation requires extracellular Ca\textsuperscript{2+} (Text-figure 7).

Changes in Ca\textsuperscript{2+} fluxes and intracellular Ca\textsuperscript{2+} may also modulate PMN degranulation\textsuperscript{9,13-15,18,22,24,26} and chemotaxis.\textsuperscript{16-20,25,32} If Ca\textsuperscript{2+} changes do modulate all three cellular responses, then factors influencing the accumulation and fluxes of Ca\textsuperscript{2+} should have similar influences on each. This is not the case. For instance, concentrations of chemotactic factors above an optimal level inhibit maximal Boyden chamber chemotaxis\textsuperscript{6,16} but do not inhibit degranulation\textsuperscript{6-8} or aggregation.\textsuperscript{27-31} Second, high concentrations of extracellular Ca\textsuperscript{2+} inhibit chemotaxis\textsuperscript{17,21} and \(\beta\)-glucuronidase release\textsuperscript{14} but enhance lysozyme release\textsuperscript{14} and aggregation (Text-figure 6). Third, cytochalasin B in low concentrations enhances chemotaxis but in high

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Influence of varying phosphate concentrations on the large particle percentage of PMN suspensions exposed to FMLP. The buffer contained 1.4 mM Ca\textsuperscript{2+} and 0.7 mM Mg\textsuperscript{2+}.}
\end{figure}
concentrations inhibits chemotaxis; this agent can only enhance, not inhibit, degranulation and aggregation (Text-figure 1). Fourth, A23187 inhibits chemotaxis but stimulates degranulation (Table 1) and aggregation (Text-figure 2). Fifth, at concentrations above \(10^{-4}\) M, La\(^{3+}\) inhibits chemotaxis, does not influence degranulation (Table 1), and induces prominent cellular aggregation (Text-figure 3); from \(10^{-6}\) M to \(10^{-5}\) M, La\(^{3+}\) does not influence chemotaxis but inhibits degranulation and aggregation (Text-figure 4). In these examples, an agent frequently inhibits chemotaxis while stimulating or enhancing degranulation and aggregation. It may be that degranulation or aggregation interferes with chemotaxis. For instance, chemotactic factors, Ca\(^{2+}\), cytochalasin B, A23187, and La\(^{3+}\), in concentrations which inhibit chemotaxis, induce or promote sustained cellular aggregation. Aggregated cells may migrate poorly. If aggregation is responsible for the inhibition of chemotaxis, then events such as Ca\(^{2+}\) fluxes and accumulations may similarly influence all three PMN responses: aggregation could limit the detection of this stimulation in chemotactic assays.
La$^{3+}$ does not stimulate Ca$^{2+}$ influx, and its ability to aggregate PMNs is independent of extracellular Ca$^{2+}$ (Text-figure 7). Having a higher valency but similar ionic radius to Ca$^{2+}$, La$^{3+}$ may bind to Ca$^{2+}$ sites on surface membranes. Our data (Text-figure 3) suggest that, in concentrations of $10^{-4}$ M to $10^{-3}$ M, La$^{3+}$ binding may overcome the repulsive forces between cells and allow the formation of intercellular adhesions. At lower concentrations, i.e., $10^{-4}$ M to $10^{-3}$ M, La$^{3+}$ binding may block Ca$^{2+}$ influxes and, thereby, aggregation (Text-figure 4) and degranulation. Why chemotaxis is uninhibited by these levels of La$^{3+}$ is unknown.

Mg$^{2+}$ appears essential for chemotactic-factor–induced and A23187-induced aggregation (Text-figures 6 and 7). Since Mg$^{2+}$ is required for PMN adherence to glass surfaces, it also may be necessary for cell–cell adherence. La$^{3+}$, which does not require Mg$^{2+}$ to aggregate cells (Text-figure 7), may substitute for Mg$^{2+}$ in this role.

Although we suggest that intracellular Ca$^{2+}$ modulates PMN aggregation, as others suggest it modulates chemotaxis and degranulation, we are aware that the available evidence does not prove this. Thus, chemotactic factors and A23187 stimulate Ca$^{2+}$ efflux, K$^+$ efflux, Na$^+$ influx and efflux, and, perhaps, the transmembrane fluxes of other unmeasured and unidentified species. Any of these events may be important in modulating PMN function. Moreover, the aggregative response occurs before

![Text-Figure 7](image_url)

**Text-Figure 7**—Influence of Ca$^{2+}$ and Mg$^{2+}$ on the aggregation index of PMN suspensions exposed to La$^{3+}$, A23187, and FMLP. Aggregation indexes induced by FMLP were also studied on PMNs treated with cytochalasin B.
the changes in ionic fluxes and accumulations. Hence, ionic fluxes and accumulations may be epiphenomena reflecting surface membrane changes or other events which are more closely related to cell function. This area requires further study.

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