Regulation of Human Neutrophil Aggregation: Comparable Latent Times, Activator Sensitivities, and Exponential Decay in Aggregability for FMLP, Platelet-Activating Factor, and Leukotriene B₄

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We have recently described a flow cytometry technique, whose sensitivity allows direct measurements of latent times before the onset of aggregation, and of rates, maximal extents, and reversibility of aggregation (J Leuk Biol 50:434, 1991). We report here that activators which stimulate sustained cellular signaling associated with increases in intracellular calcium (ionomycin) or protein kinase C activation (phorbol myristate acetate, PMA) cause complete (≥98%) and irreversible neutrophil aggregation, with latent times for the onset of aggregation inversely proportional to the activator concentration. In contrast, the receptor-specific activators leukotriene B₄ (LTB₄), formyl peptide FMLP, and platelet-activating factor (PAF) gave only partial and reversible aggregatory responses, limited by the following similar properties: latent times of 4.5 seconds ± 1.5 seconds, independent of activator concentra-

We recently described a flow cytometry technique in which rapid and direct measurements of aggregation can be carried out on fixed suspensions of isolated neutrophils. This methodology, established with FMLP as activator, identified parameters of neutrophil aggregation, including latent times between the addition of activator and the onset of aggregation, initial rates, maximal extents, and reversibility of aggregation. We now compare the effects of three different receptor-active chemoattractants: FMLP, platelet-activating factor (PAF) and LTB₄; and two intracellular signal mimetics that yield sustained activation bypassing the calcium ionophore ionomycin. These more direct studies have led to a characterization of the parameters underlying the regulation of neutrophil aggregation. We report on five properties of neutrophils that regulate and limit aggregation when they are stimulated with receptor-specific activators: delayed onset of aggregation; common broad dose-response curves with comparable limited recruitment and spontaneous reversibility of aggregation; the absence of combined activator synergism; and exponential decay in aggregability for noncaptured neutrophils. We found no evidence for neutrophil subpopulations in responses to dual activators, but variable "cross-desensitization" of FMLP- or LTB₄-induced aggregation.

MATERIALS AND METHODS

Activators. N-formyl-methionyl-leucyl-phenylalanine, phorbol 12-myristate 13-acetate (Sigma Chemical, St Louis, MO), ionomycin, and 1-O-octadecyl-2-O-acetyl-sn-glycero-3-phosphocholine (PAF-18) (Calbiochem, La Jolla, CA) were obtained commercially. LTB₄ was obtained as a generous donation from Dr A. Roback (Merck-Frost, Montreal, Quebec, Canada). LTB₄ was diluted in methanol (maximal final concentration of methanol <0.01%), FMLP, PMA, and ionomycin in dimethyl sulfoxide (DMSO; maximum final concentration <0.01%), and PAF in DMSO/ethanol (4:1) (maximum final solvent concentration <0.1%). All activator stocks were stored at −70°C and diluted in Tyrode’s buffer.

Neutrophil preparation. Citrated peripheral blood from healthy consenting adults who were not on medication was collected and
processed as previously described. Briefly, red blood cells (RBCs) were sedimented with 1% hydroxyethylcellulose (Fulka Chemicals, Ronkonkoma, NY) and the resulting leukocyte-rich plasma was layered over 60% Percoll (Pharmacia Fine Chemicals, Dorval, Quebec, Canada) and centrifuged to isolate neutrophils. Contaminating RBCs were lysed with ice-cold water and the neutrophil pellet was reconstituted to the desired cell concentration in Ca²⁺-Mg²⁺-free Tyrode’s buffer (pH 7.2) containing 0.25% human serum albumin (final). Initial cell concentrations of 8 × 10⁶/mL were used for studies of the effects of varying activator concentrations on the efficiency of aggregation, and 1.1 × 10⁷/mL for studies of the refractoriness of neutrophils to specific activators.

Sample manipulation. Four hundred eighty microliters of cell suspension at room temperature was aliquoted into a 1.5-mL cylindrical cuvette (7 mm × 45 mm) containing a stir bar (6 mm × 1 mm), and allowed to heat to 37°C for 1 minute at stir speeds of 900 rpm in a Payton aggregometer (Model 800; Payton Scientific Ltd., Buffalo, NY). Fifteen microliters of Ca²⁺-Mg²⁺ chloride solution (1.8 mmol/L Ca²⁺-0.8 mmol/L Mg²⁺ final) + activator was added to the suspension (time 0), and 30-mL subsamples were withdrawn at the desired times and fixed immediately in 250 μL of 2% ice-cold electron microscopy-grade glutaraldehyde (J.B. Electron Microscopy Services, Pointe Claire, Quebec, Canada), followed by dilution with 1,000 μL of cold flow cytometry buffer (Hematal; Fisher Scientific, Montreal, Quebec, Canada). Samples were read on the cytometer within 4 hours. For t’s (refractoriness) studies, stirring was stopped 1 second after activator addition, and restarted at the desired time (t); the first subsample taken 1 second after restarting the stirring to establish the extent of aggregation associated with the incubation period, which was normally less than 5% for all activators tested, but increased on some occasions to 20% to 30% for t’s greater than 15 seconds.

Determination of neutrophil aggregation. Neutrophil aggregation was determined by analysis of the flow times (FT) needed to measure a constant number of particles (4,000) on a FACScan cytometer (Becton Dickinson), and by analysis of the fraction of particles counted that are singlets (F₁). We have previously shown that the flow time can be used to correct for changes in the number of particles per unit volume that occur during aggregation. In addition, the fluorescent signals emitted from glutaraldehyde-fixed neutrophils are proportional to the number of neutrophils per particle, allowing simple calculations of the percentage of neutrophils that are singlets from histograms of fluorescence data. Aggregation was expressed as the percent of the neutrophil population recruited into aggregates (%PA), where

\[
\%PA = \left(1 - \frac{(FT_0 - f_0)/(FT - f_0)}{FT_0 - f_0}\right) \times 100
\]

where FT₀ and FT are, respectively, the flow time for the sample at time 0 (control sample) and at time t, and f₀ and fₜ are the fraction of the total particles counted which are singlets at times 0 and t.

Parameters of aggregation. The parameters of aggregation used in this report have been introduced previously. Briefly, these are: tₙ, the latent time in seconds before the onset of recruitment of cells into aggregates; v₁ and vₙ, respectively the initial forward rate of aggregation (%PA/s) and the rate of reversal of aggregation (%PA/min); and PAₘₙₙ, the maximal number of recruited neutrophils (%PA).

RESULTS

The aggregatory response of human neutrophils to receptor-specific and nonspecific activators. Using direct methods for evaluating neutrophil aggregation, we have recently shown that activation with maximal doses of FMLP (0.5 μmol/L) results in a single slowly reversible aggregatory response (15) (Fig 1A). We now report a family of reversible aggregation curves with decreasing FMLP concentrations (Fig 1A). In contrast to FMLP, stimulation with LT₄ (0.1 μmol/L - 1 μmol/L) leads to a rapidly reversible aggregatory burst followed by a secondary reversible burst of lower magnitude (Fig 1B). The secondary burst consistently occurred between 1.5 and 2 minutes after LT₄ addition, irrespective of the activator concentration used. Stimulation with PAF also resulted in rapidly reversible aggregatory responses (Fig 1C), with comparably limited maximal extents of aggregation to those found for LT₄ stimulation (Table 1). In addition, we found a biphasic aggregatory response for PAF-stimulated neutrophils at activator concentrations ≥500 nmol/L.

Very different results were obtained when using stable "intracellular signal mimetics." Stimulation with suprathreshold concentrations of PMA (≥5 μmol/L) leads to an irreversible aggregatory response over 7 minutes, with ≥97% of singlet neutrophils recruited into aggregates of at least two cells (Fig 1D). Similar results were found for ionomycin treatment with respect to maximal and "stable" recruitment of single cells into aggregates. However, at high ionomycin concentrations (≥50 μmol/L), cell viability as measured by Trypan Blue exclusion was reduced to zero by 3 minutes, associated with rapid deaggregation of the cells (Fig 1E).

When neutrophils were stimulated by activators that function via specific receptors on the neutrophil cell surface, the latent times before aggregation were on the order of 3 to 6 seconds, even with 1,000-fold variations in activator concentration (Fig 1). In contrast to the receptor-specific activators, a minimum of 30 seconds was necessary before the onset of aggregation stimulated by the highest concentrations of PMA evaluated (Fig 1D). In addition, lowering PMA concentrations considerably lengthened the latent time, with latent times inversely proportional to the log of the PMA concentration (not shown). As PMA concentrations were lowered, the rate of aggregation decreased, and the time necessary to reach PAₘₙₙ values of 90% to 97% increased.

We found that at constant activator concentrations of 0.5 μmol/L, the initial forward rates of aggregation stimulated by the specific activators were not significantly different (Table 1). However, the maximal extent of aggregation varied significantly with activator type, with maximal recruitment of 30% to 50% of singlets for LT₄ and PAF stimulation, and 50% to 70% singlet recruitment with FMLP (Table 1). The v₁ values for aggregation stimulated by 0.5 μmol/L LT₄ and 0.5 μmol/L FMLP for individual experiments were very similar, while PAₘₙₙ values were consistently lower for LT₄ stimulation; ratio of v₁ stimulated by LT₄ versus FMLP-0.93 ± 0.21 (mean ± SD, n = 5), ratio of PAₘₙₙ = 0.65 ± 0.13 (n = 6), P < .001, paired Student’s t-test.

As shown in Fig 1A, FMLP stimulation gave slowly reversible aggregatory responses with rates of reversal 20-fold slower than forward rates of aggregation. However, both PAF and LT₄ gave rapidly reversible aggregatory responses, with rates of reversal only threefold to fivefold

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lower than forward rates of aggregation (Figs 1B and 1C, Table 1).

We have previously shown that the time to the onset of deaggregation was dependent on the stir speed at maximal FMLP concentrations. We now show that at constant stir speeds of 900 rpm, the onset time for reversal of aggregation is also dependent on activator concentration. Thus, as activator concentration increased, so did the onset time for deaggregation (Fig 1A-C).

Table 1. Evaluation of the Effects of Receptor-Specific Neutrophil Activators on Parameters of Aggregation

| Activator | $t_1$ (s) | $v_1$ (%PA$_{max}$/s) | PA$_{max}$ (%PA$_{0}$) | $v_1$ (%PA$_{max}$/min)

| FMLP | 6.2 ± 1.2 | 3.5 ± 1.0 | 61.7 ± 9.5 | 10.2 ± 1.3
| LTB$_4$ | 6.1 ± 1.0 | 2.7 ± 0.6 | 40.3 ± 5.9 | 32.0 ± 2.2
| PAF | 4.6 ± 0.6 | 1.7 ± 1.0 | 41.8 ± 15.6 | 31.2 |

Data are summarized from different experiments for absolute values determined for 0.5-μmol/L activator concentrations. Values are means ± SD of 10 experiments for FMLP, 6 experiments for LTB$_4$, and 3 experiments for PAF, except for the value of $v_1$ with PAF stimulation, which is a mean of two experiments. Significant differences are shown for values compared with those found for FMLP.

* Values are for the first wave of aggregation only (see Fig 1B).
1 $P < .001$, Student's t-test.
2 $P < .01$, Student's t-test.

Sensitivity of neutrophil aggregation to different activators. Dose-response studies yielding the concentration of activator leading to one half of maximal initial forward rates of aggregation ([Act]$_{1/2}$) were performed (Figs 1 and 2). We found that for all the activators tested that function via specific receptors on the neutrophil cell surface, the threshold concentrations required for measurable rates of aggregation were on the order of 0.5 nmol/L to 1 nmol/L, with [Act]$_{1/2}$ values, expressed on a molar basis, of $\approx 25$ nmol/L: 21 ± 9 nmol/L, 24 ± 10 nmol/L and 35 ± 27 nmol/L (n = 3), respectively, for FMLP, LTB$_4$, and PAF as activators. Identical results in the log dose-response curves were obtained for the maximal extent of neutrophil aggregation (PA$_{max}$) measured for the first wave of aggregation (Fig 1) for all three activators (not shown). We found that the stir speed used during the aggregation assay had no effect on the [Act]$_{1/2}$ stimulated by FMLP, as identical values were found at 900 rpm and 300 rpm (not shown). Although PMA acts by diffusing into the cells, its extracellular concentration clearly affects the rate of neutrophil aggregation, with an [Act]$_{1/2}$ of 10 ± 4 nmol/L (n = 4). However, unlike the receptor-specific activators, the log dose-response curve for PMA-induced aggregation shows a threshold effect, rather than a continuous concentration dependent effect (Fig 2).

Ionomycin showed a similar threshold effect, with onset and maximal aggregation occurring between $\approx 1$ and 10 μmol/L (Fig 1E).
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Fig 2. Dose-response curves for different neutrophil activators. The Y-axis represents the relative forward rate of aggregation as a function of the highest forward rate of aggregation for any one experiment. The absolute values for maximal initial forward rates of aggregation (v_f) stimulated with 0.5 μmol/L activator are presented in Table 1. Data points are means of 2 to 5 experiments.

Time-dependent refractoriness of aggregation responses. To study whether the reversible aggregation stimulated by receptor-specific activators was associated with a time-dependent loss in the neutrophils' ability to aggregate, activator was added to stirred suspensions of neutrophils and stirring was stopped after 1 second for different times (τ) before restarting the stir (Fig 3). We found that τ times resulting in 50% of maximal initial rates or extent of aggregation, when compared with control preparations (τ = 0), were on the order of 15 to 30 seconds, with 90% refractoriness at 30 seconds or greater (Table 2). Also, we found that both the initial forward rate of aggregation (v_f) and the maximal extent of aggregation (P_{Amax}) decayed exponentially with increasing τ times, with an extrapolated average delay of ≈4

![Graph](image)

Fig 3. Examples of the effects of the time between the addition of activator and the onset of stirring (τ), on the aggregatory response to receptor-specific activators. The curves are representative of at least three donors for neutrophil suspensions stimulated with (A) 0.5 μmol/L FMLP, or (B) 0.5 μmol/L LTB₄.
seconds before the exponential decay began (Fig 4). In addition, the individual slopes of \(v_f\) versus \(\tau\) were identical for different activators (FMLP and LTB\(_4\)) when examined for the same donor on the same day (not shown).

We attempted to overcome the refractoriness found with 0.5 \(\mu\)mol/L FMLP at \(\tau = 30\) seconds, by adding 10 times the concentration immediately before commencing stirring. However, even with 10-fold increases in the concentration of activator, the cells remain in a refractory state.

Finally, because lower stir rates of neutrophil suspensions have previously been shown to yield more stable, less reversible aggregation,\(^1\)\(^5\) we compared refractoriness for FMLP (0.5 \(\mu\)mol/L) at 900 rpm and 300 rpm for the same speed were found to be identical (not shown).

Effects of combined or successive additions of two different receptor-specific activators (LTB\(_4\) and FMLP). It has previously been suggested from light transmission studies that successive additions of two activators, such as FMLP and LTB\(_4\), can yield two waves of aggregation with apparent recruitment of neutrophil subpopulations.\(^1\)\(^2\) We tested whether adding FMLP together with, or at peak aggregatory response to another activator (LTB\(_4\)), leads to a potentiation of the aggregatory response, or to a second phase of aggregation. We were unable to find a potentiation of the initial rate or maximal extent of aggregation when 0.5 \(\mu\)mol/L FMLP and 0.5 \(\mu\)mol/L LTB\(_4\) were added together (not shown).

However, under these conditions 80% of singlet neutrophils were recruited into aggregates with FMLP addition alone. Therefore, we stimulated neutrophils with 10 nmol/L or 20 nmol/L LTB\(_4\) or FMLP alone or simultaneously. Adding 10 nmol/L of LTB\(_4\) and FMLP together gave maximal extents of aggregation that were considerably less than additive when compared with PA\(_{max}\) values found with 10 nmol/L LTB\(_4\) or 10 nmol/L FMLP alone, and that corresponded with those found with 20 nmol/L FMLP alone (Fig 5A). In addition, adding 10 nmol/L FMLP after maximal aggregation stimulated by 10 nmol/L LTB\(_4\) resulted in a second burst of aggregation similar to the response seen with 10 nmol/L FMLP alone, i.e., an expected further recruitment yielding up to 50% PA\(_{max}\) was not observed (Fig 5B).

The above data (especially that in Fig 5B) suggests that the refractoriness observed for each activator added alone (Fig 4) was specific for that activator. In initial experiments, this was confirmed with FMLP and LTB\(_4\), where minor "cross-desensitization" was observed when neutrophil suspensions were allowed to reversibly aggregate to LTB\(_4\) or were incubated with LTB\(_4\) for 7 minutes before the addition of FMLP (Fig 6, and group I of Table 3). However, examination of additional donors showed both poor and effective desensitization depending on the donor, as well as the concentrations of LTB\(_4\) and FMLP used (Table 3). Thus, for 4/6 donors, little to no desensitization was found when cells were treated with 500 nmol/L LTB\(_4\) for 7 minutes followed by 500 nmol/L FMLP, and in 2/6 donors major desensitization did occur. However, in one donor that showed desensitization when 500 nmol/L LTB\(_4\) was added first, no desensitization was observed if only 50 nmol/L LTB\(_4\) was added before the FMLP. Conversely, a donor that showed minimum desensitization when 500 nmol/L FMLP was added after either concentration of LTB\(_4\) used, did show desensitization if a lower concentration (50 nmol/L) of FMLP was added (Table 3). Thus, there can be significant donor variability when these desensitization studies are performed.

**DISCUSSION**

Flow cytometry was used to study human neutrophil aggregation dynamics, which showed comparable limited re-
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Fig 5. The dynamics of the aggregatory response with simultaneous and consecutive addition of LTB4 and FMLP. (A) The effect of the simultaneous addition of 10 nmol/L FMLP and 10 nmol/L LTB4 on the dynamics of aggregation. (B) The effect of adding 10 nmol/L FMLP to a neutrophil suspension undergoing an aggregatory response to 10 nmol/L LTB4. All activator concentrations are final molarity.

sponses to the receptor-specific activators; FMLP, PAF, and LTB4. Our results found with flow cytometry are similar to those found by others using electronic particle counting. However, flow cytometry has allowed us to study neutrophil aggregation in greater detail, uncovering five properties shared by aggregating neutrophils stimulated with receptor-specific activators. These aggregation properties include, (1) similar latent times preceding aggregation; (2) comparable activator concentrations driving aggregation over a broad dose response; (3) an exponential decay in aggregability of noncaptured activated neutrophils; (4) an activator type- and concentration-dependent reversibility of aggregation; and (5) the absence of synergistic effects when combining two activators. These limits were not observed for the stable “intracellular signal mimetics” PMA and ionomycin, which yielded “irreversible” aggregation of all neutrophils above a critical threshold and over a narrow range of concentrations.

All the receptor-specific activators tested gave latent times (t_l) preceding aggregation of 3 to 6 seconds, independent of activator concentration. In contrast, the latent times for PMA- and ionomycin-stimulated aggregation were concentration-dependent, decreasing exponentially with increasing PMA concentration. Latent times for the onset of superoxide production have also been reported to be activator concentration-independent for FMLP stimulation and
concentration-dependent for PMA stimulation.\textsuperscript{18,19} Differences in latent times with changes in the concentration of PMA or ionomycin may represent the time needed for the diffusion of a threshold concentration of activator into the cell before a functional response can be elicited, as suggested by the steep dose-response curve for PMA (Fig 2). The latent time for neutrophil aggregation of 3 to 6 seconds is in marked contrast to the 1 second latent time reported for platelet aggregation.\textsuperscript{14}

The receptor-specific activators PAF and LTB\textsubscript{4} caused significantly lower maximal extents of aggregation than did FMLP. The ability of FMLP to recruit more neutrophils into aggregates than PAF or LTB\textsubscript{4} may be related to the extent of activation signals stimulated by each of these activators.\textsuperscript{20} LTB\textsubscript{4} is reported to induce activation signals such as internal calcium transients that are short-lived compared with those induced by formyl peptides.\textsuperscript{21,22} Our aggregation results suggest that PAF stimulation induces activation signals that are similarly, if not more, short-lived as those induced by LTB\textsubscript{4}.

PMA is known to stimulate sustained activation of protein kinase C.\textsuperscript{16} The recruitment of most neutrophils into stable aggregates upon PMA treatment (\(\geq 5\) nmol/L) may be related to sustained stimulation of phosphorylation activity. The \(\alpha\) subunits of the adhesion glycoproteins Mac-1 (\(\alpha\text{M/CD11b}\)) and p150,95 (\(\alpha\text{J/CD11c}\)) have been shown to be constitutively phosphorylated in neutrophils.\textsuperscript{23,24} In contrast, \(\beta\) subunits (\(\beta_2\)) become minimally phosphorylated with FMLP activation, or strongly phosphorylated with PMA activation.\textsuperscript{23,25} Thus, the level of phosphorylation of the \(\beta_2\) subunit appears to be involved in mediating reversible and irreversible aggregatory responses. In addition, sustained elevated intracellular calcium, expected with ionomycin stimulation,\textsuperscript{17} also caused complete recruitment of all neutrophils. Although phorbol esters and calcium ionophores are not "physiologic" activators, the use of these activators shows that all neutrophils are able to aggregate. Thus, the limited aggregation found with receptor-specific activators cannot be attributed to the existence of subpopulations of neutrophils that are unable to aggregate. The ability to recruit all neutrophils into aggregates using receptor-specific activators appears to be related to the rapid refractoriness associated with these stimuli, which is not observed with PMA nor ionomycin.

Analyses of dose-response data for FMLP- and LTB\textsubscript{4}-induced "aggregation" measured as maximal changes in light transmission\textsuperscript{8,11-13} yield apparent \([\text{Act}]_{1/2}\) values of 6 to 10 nmol/L and 1 to 2 nmol/L, respectively. These are up to 20

\begin{table}[h]
\centering
\caption{Neutrophil Desensitization to FMLP-Induced Aggregation by Pre-Incubation With LTB\textsubscript{4}}
\begin{tabular}{lcccc}
\hline
 & \multicolumn{2}{c}{LTB\textsubscript{4}} & \multicolumn{2}{c}{50 nmol/L} \\ \cline{2-5}
 & 500 nmol/L & 50 nmol/L & 500 nmol/L & 50 nmol/L \\ \hline
FMLP & \multicolumn{4}{c}{Percent Desensitization\textsuperscript{*}} \\ \hline
Group I & 25 & 40 & 26 & 26 \\
 & 75 & 75 & 0 & 70 \\
Group II & 85 & 75 & 39 & 0 \\
 & 62 & \multicolumn{2}{c}{--} & 70 \\
\hline
\multicolumn{5}{l}{* \%PA\textsubscript{max} was measured for the FMLP concentrations shown 7 min after LTB\textsubscript{4} addition, and the values were compared with \%PA\textsubscript{max} levels found when neutrophils were stirred for 7 min in the absence of LTB\textsubscript{4}, and FMLP subsequently added.}
\multicolumn{5}{l}{\textsuperscript{1} Groups I and II correspond to donors showing poor and very good desensitization to 500 nmol/L LTB\textsubscript{4} followed by 500 nmol/L FMLP.}
\end{tabular}
\end{table}
times lower than our estimates from direct measurements of aggregation, especially for results with LTB₄ (Fig 2). These lower values observed from light transmission studies are predicted to reflect the contribution of intracellular changes such as actin polymerization and degranulation on light transmission responses.⁴ In this regard, threshold concentrations and [Act]₁/₂ values have been reported to vary with the neutrophil functional response measured.²⁶ A direct relationship between the dissociation constant (kd) and [Act]₁/₂ for the chemotactic responses of neutrophils to LTB₄ has been reported.²⁷ It is perhaps significant that the [Act]₁/₂ values of ~20 nmol/L observed for FMLP, PAF, and LTB₄ are identical to the low-affinity kd reported for FMLP, and similar to kd values reported for PAF and LTB₄.²⁸,²⁹ These results suggest that similar receptor occupancy and capacity for driving neutrophil aggregation occurs with any of these three activators.

It has been reported that the "aggregatory" response, measured from changes in light transmission (ΔT), to FMLP added at the peak response to LTB₄, yields a further ΔT response equal to that found in the independent response to FMLP, and that successive subpopulations of neutrophils are recruited by these two activators.¹³ We found no evidence for additional recruitment of neutrophil subpopulations in response to FMLP and LTB₄ (Fig 5). Because these activators show identical log dose-response curves and [Act]₁/₂ values, the extent of aggregation appears limited by simple additive effects of the activator concentrations. The absence of synergistic or amplified responses to two activators in contrast to the synergism found for platelet microparticles.³⁰

We found identical exponential decay in neutrophil aggregability for all three activators evaluated (FMLP, PAF, and LTB₄) pointing to a common mechanism driving deaggregation. This behavior of activated neutrophils in the absence of collisions stands in marked contrast to the absence of such refractoriness for platelet microparticles formation.¹⁴ It is predicted that the expression of an activated Mac-1 adhesion molecule (CD11b/CD18) on neutrophils will be reversible with a time course paralleling that of reformation. Alternatively, the regulation (shedding) of L-selectin may play a role in the observed refractoriness.³¹

We found variable evidence for "cross-desensitization" of FMLP-induced aggregation by prior desensitization with LTB₄, whether occurring in the absence or presence of fully reversible aggregation. Depending on the donor, refractoriness can be receptor-specific, at least for the LTB₄/FMLP combination studied to date. It is clear from Table 3 that there is diversity in the responses of neutrophils from various donors to desensitization with combinations of LTB₄ and FMLP. Preliminary studies on the levels of L-selectin shedding following LTB₄ addition, measured by flow cytometry with FITC-Leu-8 antibody, did not show any correlation with the degree of desensitization that followed when FMLP was added. A complete understanding of such cross-receptor effects will require the appropriate use of blockers for receptors and synthesis of PAF and LTB₄, as it is known that one activator (ex: FMLP) can cause activated neutrophils to synthesize other activators such as PAF and LTB₄.³⁵-³⁷ Such cross effects are suggested by the second waves for aggregation seen with PAF and LTB₄ (Fig 1).

In summary, using direct counting of neutrophil particles by flow cytometry to measure aggregation, we have found five properties of neutrophils that appear to regulate and limit aggregation when stimulated with three receptor-specific activators: delayed onset of aggregation; common broad dose-response curves with comparable limited recruitment and spontaneous reversibility of aggregation; the absence of combined activator synergism; and exponential decay in aggregability for noncaptured neutrophils. These neutrophil properties will limit the potentially harmful effects of aggregated neutrophils on vascular endothelium, which have been directly linked to ischemic complications in both cerebrovascular and cardiovascular settings.⁶,⁷ Mechanisms limiting intravascular neutrophil aggregation would limit degranulation and endothelial injury, while reducing the likelihood of blood-flow obstruction at inflammatory sites, permitting a continued influx of neutrophils where they are needed.⁵

REFERENCES

12. Ringertz B, Palmblad J, Lindgren JA: Stimulus-specific neu-


34. Simon SI, Chambers JD, Butcher E, Sklar LA: Neutrophil aggregation is β2-integrin and L-selectin-dependent in blood and isolated cells. J Immunol 149:2765, 1992


Regulation of human neutrophil aggregation: comparable latent times, activator sensitivities, and exponential decay in aggregability for FMLP, platelet-activating factor, and leukotriene B4

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