Activation Mechanisms of Adherent Human Neutrophils

By Irene Ginis and Alfred I. Tauber

The mechanism by which unstimulated human neutrophils initiate a respiratory burst on adherence to a surface has been examined. When neutrophils adhere to a plastic surface, they immediately generate a sustained burst of superoxide (O$_2^-$). However, this respiratory burst is not initiated by adherence alone, since neutrophils attached to fibronectin fail to mount a response. Adhesion to plastic is calcium (Ca$^{2+}$) independent, but O$_2^-$ production requires Ca$^{2+}$-containing buffer in the initiation phase, that is, during adhesion and the early phase of O$_2^-$ production. The Ca$^{2+}$-dependent step was shown to involve protein kinase C (PK-C) in that the O$_2^-$ production, but not adherence, was blocked with 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), and PK-C was found to translocate from the cytosol to the membrane on adhesion. Furthermore, it may be inferred that this translocation is the result of the generation of a Ca$^{2+}$-independent form of PK-C, PK-M, since leupeptin, which inhibits the generation of PK-M, also blocked O$_2^-$ production. This finding was corroborated by showing that after 5 minutes in a Ca$^{2+}$-containing buffer, enough time to initiate O$_2^-$ production and PK-C translocation, Ca$^{2+}$ is no longer required for sustained O$_2^-$ release. These results, in aggregate, demonstrate that neutrophils are activated by adhesion to plastic to generate O$_2^-$, a PK-C-dependent process that appears to involve a Ca$^{2+}$-independent form of the kinase, PK-M. Why adherent neutrophils generate a respiratory burst on plastic and not fibronectin surfaces probably reflects activation of distinct receptors, whose nature must still be defined. Another issue to address is the priming effect of adhesion, since cells adherent to plastic or fibronectin-coated surfaces have an enhanced O$_2^-$ response to formylmethionyl-leucine-phenylalanine (FMLP) compared with neutrophils stimulated in suspension. This may relate to increased Ca$^{2+}$ mobilization, an important mediator of priming for FMLP responses. Thus, adhesion as a priming event does not necessarily initiate cell effector function, and the further elucidation of the plastic and fibronectin models suggests a means of characterizing the crucial events that control neutrophil activation.

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times as indicated under the same conditions. The aspirated buffer from each dish was then centrifuged (400g for 5 minutes) and cytchrome C reduction was assessed by scanning between 570 and 530 nm in a Perkin Elmer Model 559 spectrophotometer.

The amount of O$_2^-$ production initiated in adherent cells by the addition of unpopsonized zymosan (2.0 mg/mL), chlamydia (50 organisms/cell), or FMLP (5 x 10$^{-7}$ mol/L) followed the above protocol except that the agonists were added 10 minutes after cells were allowed to adhere and nonadherent cells were washed out.

The rate of O$_2^-$ production in adherent neutrophils stimulated with FMLP was measured by the SOD-inhibitable reduction of cytchrome C by continuously monitoring optical density at 550 nm by minor modification of previously described methods. A total of 5 x 10$^6$ in 1 mL PBS$^+$ neutrophils was allowed to adhere for 5 minutes on the opaque wall of a 4-mL, two-sided polystyrene cuvette (Fisher Scientific Co, Pittsburgh, PA), washed, and incubated with 1.8 mL PBS$^+$ and 1.5 mg/mL cytchrome C. Absorbance was monitored under continuous stirring (Spectrocell, Inc, Oreland, PA) and thermostatted at 37°C. After a preincubation period of 3 minutes, during which no spontaneous metabolic activity was observed, neutrophils were stimulated with FMLP at a final concentration of 5 x 10$^{-7}$ mol/L. Note that cytochalasin B was not used in these studies. Rates of O$_2^-$ production during the first 2 minutes following stimulation were calculated from the original tracings.

**Neutrophil adherence.** The number of adherent cells was measured individually for each experiment. Cuvettes or culture dishes with adherent cells were washed three times with PBS$^+$ to remove nonadherent cells and incubated with Pierce BCA protein assay reagent (Pierce, Rockford, IL) for 30 minutes at 37°C. Each sample was then read at 562 nm (Beckman DU-7 spectrophotometer, Beckman Instruments, Fullerton, CA) and compared with a standard protein curve derived from known cell numbers. As shown in Fig 1A, in 10 minutes, 2.5 to 3.2 x 10$^6$ cells adhered to the surface (50% to 65% of the total number of cells adhered). In fibronectin experiments, Petri dishes or cuvettes were coated with 3 pg/cm$^2$ of standard protein curve derived from known cell numbers. As shown in Fig 1A, in 10 minutes, 2.5 to 3.2 x 10$^6$ cells adhered to the surface (50% to 65% of the total number of cells adhered). In fibronectin experiments, Petri dishes or cuvettes were coated with 3 pg/cm$^2$ of human plasma fibronectin according to Fehr et al, allowing to incubate for 45 to 60 minutes at room temperature, washed twice with PBS$^+$, and immediately used, as described above. The number of adherent cells was 2.7 to 3.4 x 10$^6$.

In these experiments, a model was designed in which cells were first allowed to adhere and were then activated with FMLP to assess each process independently. In most previous studies, an agonist was added to neutrophil suspensions, then cells were allowed to adhere, which not only initiated neutrophil end function responses, but also greatly enhanced adherence. Under such conditions, neutrophils adhered in high proportion. In our preliminary experiments, we showed that adherence of cells without stimulation is dependent on the type of surface used, while O$_2^-$ release stimulated by FMLP was equal on each surface when calculations were corrected for the number of adherent cells (data not shown), which alerted us to calculate neutrophil response (ie, O$_2^-$ generation) on the basis of adherent cell number.

**Measurement of intracellular calcium [Ca$^{2+}$/].** [Ca$^{2+}$/] levels were determined using the fluorescent indicator (Fura-2). Isolated neutrophils were incubated with the acetoxy methyl ester of Fura-2 (2.5 umol/L) in PBS$^+$ for 30 minutes at 37°C, washed, and resuspended in fresh buffer; 2 mL (10$^6$/mL) of Fura-2 loaded cells were constantly stirred in the cuvette for [Ca$^{2+}$/], measurements of suspended cells, or cells were first allowed to adhere to the cuvette wall as described for continuous O$_2^-$ production and then incubated with 2 mL of fresh buffer. In each case, cells were stimulated with 5 x 10$^{-7}$ mol/L FMLP. Ca$^{2+}$/ determinations were measured using the ratio of the peak fluorescence at excitation wavelengths of 340 to 380 nm and emission wavelength of 510 nm (Perkin-Elmer [Norwalk, CT] model LS5 spectrofluorometer). The Ca$^{2+}$/ concentration was calculated using a Kd of 224 nm.

**PK-C translocation.** A 4-mL neutrophil suspension (5 x 10$^6$ mL) was preincubated for 10 minutes with 1 mmol/L leupeptin and then added to two Petri dishes (100 x 15 mmol/L) and allowed to incubate at 37°C for 5 minutes. Nonadherent cells were washed out, and 1 mL of fresh PBS$^+$ containing 1 mmol/mL leupeptin was added. Neutrophils were then removed with a cell scraper (Giboware, Gibico, Grand Island, NY), paired samples were pooled, and the distribution of PK-C in cytosol and membrane fractions was then assessed according to the method of Melloni et al. In summary, cells were centrifuged for 7 minutes at 4°C, 400g, and the pellet was resuspended by vortexing in 1 mL of lysis buffer (10 mmol/L Heps, 0.25 mol/L sucrose, 5 mmol/L EDTA, 1 mmol/L dithiothreitol) containing 1 mmol/mL leupeptin. The suspension was then gently sonicated three times for 10 seconds at 4°C using a tip sonicator (Ultrasonics, Maidstone, England) at settings 3 to 5 and transferred to a polycarbonate tube and centrifuged at 150,000g for 30 minutes, thus obtaining cytosolic and membrane fractions. The membrane fraction was solubilized by resuspending the pellet in 1 mL of lysis buffer containing 1 mmol/mL leupeptin and 0.1% Triton X-100, thoroughly homogenized at 4°C, and then incubated in an ice bath for 1 hour. The harvested cytosolic and solubilized fractions, respectively, were loaded onto a 1.5-mL packed E-52 resin (What-
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man) previously equilibrated with 10 mmol/L Hepes, 5 mmol/L EDTA, 1 mmol/L dithiothreitol, pH 7.5. The kinase activity was eluted from resin with pH 7.5.

For measurements of cytosolic and membraneous PK-C activity, 20 μL of each sample were incubated for 10 minutes at 30°C with 30 μL of the PK-C incubation mixture, containing 11.1 mmol/L Tris buffer (pH 7.5), 0.17 mmol/L ATP, 1.3 mg histone, 67 μg phosphatidyl-L-serine, 2.0 μg phorbol dibutyrate, 16.7 mmol/L MgCl₂, 1.6 mmol/L CaCl₂, and 5 μmol/L [α-32P]ATP. For control experiments, 5 mmol/L EDTA substituted for Ca²⁺ and phospholipids. After incubation at 30°C for 10 minutes, 30-μL samples were pipetted onto filter paper (1.5 cm²), which were then immediately transferred to a solution containing 10% TCA and 10 mmol/L sodium pyrophosphate. The filters were then washed four times in this solution, dried, and counted in Scintiverse Universal LSC cocktail (Fisher Scientific, Fairlawn, NY); the radioactivity was measured with a TM Analytic Scintillation (Brandon, FL) counter. Background radioactivity of the sample without 32P was subtracted from the radioactivity of each probe. The difference between the radioactivity of the probe incubated in the presence of phospholipids and Ca²⁺ and that of the control probe incubated without phospholipids and Ca²⁺ was considered a measure of PK-C activity in each sample. As all the samples were obtained from the same number of cells (2 to 3 × 10⁶), it was assumed that the ratio of radioactivities from membrane and cytosol aliquots of each sample reflected the distribution of PK-C activity in the cells studied.

RESULTS

Adherent neutrophil O₂⁻ production. When neutrophils become adherent to plastic surfaces, "spontaneous" O₂⁻ is generated. As shown in Fig 1A, adhesion becomes stable by 5 to 10 minutes, while O₂⁻ production does not plateau until 40 minutes of incubation (Fig 1B). Note that the magnitude of O₂⁻ production is similar to that obtained with FMLP-stimulated neutrophils in suspension (see Fig 4A). Cells adherent to fibronectin, however, generated no spontaneous O₂⁻ (Table 1).

Adhesion on a plastic surface is Ca²⁺ independent, for there was no difference observed in adhesion between cells incubated in PBS²⁻, Ca²⁺-free PBS with EGTA (3 mmol/L) (Fig 2), or with Ca²⁺-depleted cells (incubated for 2 hours in Ca²⁺-free buffer)²⁻: 3.2 ± 1.3 × 10⁶ cells for each case (mean ± SD for four experiments). However, O₂⁻ stimulated by adherence is diminished in a Ca²⁺-free buffer (Fig 3). During the first 10 minutes of incubation, O₂⁻ production was observed in Ca²⁺-free medium, but the burst was not sustained as observed in PBS²⁺. This finding suggested a role for extracellular Ca²⁺ for continued respiratory burst activity, and we thus directed our attention to PK-C, whose sustained activation is dependent on an extracellular source of Ca²⁺.

Specifically, having shown that adherence was Ca²⁺ independent, but subsequent O₂⁻ generation was Ca²⁺ dependent, we next determined whether PK-C might regulate either function.

Predictably, PK-C would have no role in the adhesion reaction, but might participate in the activation of the respiratory burst. Our first approach was to use the kinase inhibitor H-7. Neutrophils were preincubated with various concentrations of H-7 for 5 minutes and adhesion and spontaneous O₂⁻ production was then assessed. Neutrophil adhesion was unaffected by the inhibitor (Fig 2), although H-7 effectively inhibited O₂⁻ production, with significant inhibition obtained with 30 μmol/L (P < .04). When another PK-C inhibitor, staurosporin, was used, O₂⁻ production was inhibited more than 90% at concentrations of approximately 40 to 80 nm, but adherence was unaltered at this concentration (data not shown). Because the staurosporine inhibitory effect on O₂⁻ generation required higher concentra-

Table 1. O₂⁻ Production of Stimulated Human Neutrophils on Plastic or Fibronectin-Coated Surfaces

<table>
<thead>
<tr>
<th>Surface/Stimulus</th>
<th>None</th>
<th>Zymosan</th>
<th>Chlamydia</th>
</tr>
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<tbody>
<tr>
<td>Plastic</td>
<td>5.08 + 2.16 (11)</td>
<td>7.56 + 1.82 (8)</td>
<td>2.81 + 1.24 (5)</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>1.59 + 1.05 (6)</td>
<td>8.36 + 7.4 (3)</td>
<td>0.96 + 1.03 (3)</td>
</tr>
</tbody>
</table>

Data are expressed as nmol O₂⁻/10⁶ cells/15 minutes (mean ± SD). Numbers in parentheses indicate number of experiments.

Probability values: a v b, <.05; a v c, <.05; a v d, <.01; d v e, <.01; and d v f, not significant.
tions than expected as a result of its exclusive effects on PK-C. We could only conclude that these data suggested that adhesion was PK-C independent, but that resultant O$_2^-$ generation was sensitive to kinase inhibition.

PK-C activation is a multistep process and only its first stage, translocation of the phospholipid-dependent form of PK-C from the cytoplasm to the cell membrane, requires Ca$^{++}$. Following thiol proteinase (calpain) cleavage of PK-C, a membrane-free form is generated that is both Ca$^{++}$ and phospholipid independent. This schema might explain how activation of native PK-C or, alternatively, elaboration of the Ca$^{++}$-independent PK-M form might mediate this O$_2^-$-generating activity. PK-M activity would be independent of Ca$^{++}$ and sensitive to the calpain inhibitor, leupeptin.

To examine this hypothesis, we measured O$_2^-$ production in neutrophils that were first allowed to adhere in PBS$^+$, then nonadherent cells were washed out and fresh Ca$^{++}$-free buffer was added to the dishes for a 10-minute incubation. The results, shown in Table 2, demonstrate that preincubation of cells in PBS$^+$ buffer for 5 to 10 minutes (a), is sufficient for subsequent O$_2^-$ production, even in a Ca$^{++}$-free buffer, comparable to the case in which Ca$^{++}$ is present in both steps (b). If the first incubation was performed in Ca$^{++}$-free buffer (c), in a Ca$^{++}$-containing buffer with H-7 (d), or in Ca$^{++}$-containing buffer with the calpain inhibitor-leupeptin (e), the ability of adherent cells to produce O$_2^-$, even in the presence of Ca$^{++}$, was significantly diminished or completely abolished. Leupeptin had no significant effect on adhesion (data not shown).

To exclude the possibility of a nonspecific effect of H-7 or leupeptin, and to directly confirm PK-C activation on adherence to plastic, direct measurements of PK-C activity in membrane and cytosol of adherent neutrophils were undertaken. As shown in Table 3, neutrophils, either kept in suspension or allowed to adhere for 5 minutes, exhibited significant differences in membrane-associated PK-C. The possibility that PK-C translocation might be due to cell disruption was excluded by the fact that in fibronectin-adherent cells, no PK-C translocation was observed (Table 3). Thus, these results are consistent with the inhibitor and Ca$^{++}$ depletion studies.

Comparison of adherent and suspended neutrophil activation. Having shown that neutrophils generate O$_2^-$ on adherence, we then examined the effect of further stimulation with FMLP. To compare kinetics of FMLP stimulation in adherent and suspended neutrophils, O$_2^-$ was measured in a continuous assay, where activation conditions were the same (Fig 4). The duration of the FMLP-stimulated response was short in both adherent and suspended cells (2 to 3 minutes), but the rate of O$_2^-$ production was significantly higher in adherent cells to either plastic or fibronectin (Fig 4a, c, and e).

Requirements for Ca$^{++}$ by FMLP-stimulated cells and the intracellular Ca$^{++}$ mobilization response were next assessed. Adherent neutrophils showed less dependence on extracellular Ca$^{++}$ than suspended cells. Neutrophils stimulated in a Ca$^{++}$-free buffer (PBS without Ca$^{++}$ plus 3 mmol/L EGTA) exhibited a significant O$_2^-$ response, while suspended cells generated no significant quantities of O$_2^-$ under these conditions (Fig 4b, d, and f).
When FMLP-induced changes in intracellular Ca\(^{2+}\) concentration were monitored in suspended and plastic-adherent cells loaded with Fura-2 (Fig 5), the same pattern of response was seen: the Ca\(^{2+}\) increase in adherent cells was higher than in suspended cells and did not depend significantly on the presence of extracellular Ca\(^{2+}\). Further, while in suspended cells, Ca\(^{2+}\) rapidly returned to baseline levels; in adherent cells, Ca\(^{2+}\) remained elevated for a minimum of 10 minutes.

We note that the magnitude of the O\(_2^-\) response to FMLP of cells adherent to plastic for 30 minutes is less than that elicited by FMLP when cells have been adherent on a surface for 5 minutes (2.53 ± 0.964 vs 6.45 ± 1.24). Since adhesion actually leads to cell activation, this diminished response is functionally deactivation\(^{20}\) which, in this system, still requires definition. These data may well explain the results of others who found that O\(_2^-\) generation of stimulated neutrophils was diminished on surfaces.\(^{10,21}\)

As noted above, when cells adhere to a fibronectin-coated surface, no significant production of O\(_2^-\) is seen, but when these fibronectin-adherent cells are stimulated with unopsonized zymosan after 5 minutes of adhesion, significant O\(_2^-\) generation ensues (Table 1). It is noteworthy that these unopsonized particles elicit no O\(_2^-\) from human neutrophils in suspension (data not shown and refs 22 and 23) and, in this regard, adherence to either uncoated plastic or fibronectin is functionally equivalent to priming.\(^{20}\) Note, however, that unopsonized chlamydia evoked no respiratory burst in adherent cells on fibronectin and significantly inhibited O\(_2^-\) production of plastic bound cells, as previously shown for cells in suspension, confirming the organism's ability to inhibit the NADPH-oxidase.\(^{12}\)

**DISCUSSION**

Neutrophils circulate in the blood for a short period and then function primarily adherent to tissue surfaces,\(^{1-3}\) where their toxic products may be directed at sites of attachment.\(^{24-26}\) Most studies that have examined neutrophil activation characteristics have been performed with cells in suspension; however, we are concerned that cell responses may be altered by the adhesion process itself. In fact, we have found that when neutrophils adhere to plastic, they immediately initiate O\(_2^-\) production (Fig 1B). Responses to soluble or particulate stimuli reflect differences of the activation process in adherent and suspended cells. Unopsonized zymosan, which elicits no O\(_2^-\) generation by suspended neutrophils, induces a rigorous O\(_2^-\) response in cells adherent to fibronectin (Table 1). In this setting, adhesion to fibronectin may be viewed as priming.\(^{20}\) The priming effect of adherence was confirmed in the recent studies of Neumann and Kownatzki on neutrophils adherent to the nylon fibers, which also showed higher response to different activators of respiratory burst.\(^{27}\) In addition, fibronectin receptor activation on adherence might play a specific role in this priming phenomenon.\(^{28}\) Again, with zymosan or FMLP stimulation of cells bound to plastic, a less dramatic, but significant, stimulation is observed in the early adhesive phase (Table 1 and Fig 4). (In the case of chlamydia, inhibition is in fact noted, because the organism directly inhibits the respiratory burst enzyme, NADPH-oxidase.\(^{12}\)) However, when cells are allowed to adhere for longer periods (ie, 30 minutes), less O\(_2^-\) is generated by FMLP stimulation, a case of functional deactivation. The nature of the "priming" and "deactivation" phenomena was not primarily the subject of these studies, but elucidation of this observation is important in establishing the parameters by which adherent neutrophils respond to physiologic stimuli, and is the focus of ongoing studies in our laboratory.

The mechanism of O\(_2^-\) activation by adherence has been defined in these studies as involving a Ca\(^{2+}\)-independent adherence step that is independent of kinase activation (ie, insensitive to H-7 or staurosporine), and then followed by a Ca\(^{2+}\)-dependent event that initiates O\(_2^-\) production. This Ca\(^{2+}\)-dependent step appears to be the activation of PK-C, as it is H-7 sensitive and correlates with the translocation of PK-C from the cytosol to the membrane, as seen in PMA-stimulated neutrophils in suspension.\(^{29-32}\) PK-C translocation leading to cell activation, ie, O\(_2^-\) production, is blocked by leupeptin, an inhibitor of calpain (the protease that elaborates PK-M), the Ca\(^{2+}\)-independent form of PK-C.\(^{37}\) We thus hypothesized that a similar scheme of PK-C metabolism to that observed with PMA-stimulated neutrophils in suspension occurs in adherent neutrophils. Specifically, we hypothesize that PK-M is responsible for the sustained O\(_2^-\) production initiated by the adhesion event.

Finally, what is the mechanism by which adhesion initiates PK-C translocation leading to cell activation, ie, O\(_2^-\) production? By our model, adhesion alone initiates PK-C translocation, which, in other systems, is related to an increase in cytosolic Ca\(^{2+}\) and, presumably, the elaboration of diacylglycerol.\(^{17}\) However, the mechanism of this activation has not yet been defined for adherent neutrophils. In our model, when neutrophils were preincubated with PT (500
and microtubule assembly of human polymorphonuclear leukocytes. A similar pathway for PT-insensitive Ca$^{2+}$-dependent, PT-insensitive pathway has been well described, indicating that this pathway involves the activation of phospholipase D. The question as to whether adhesion molecules, or other receptors, directly trigger events leading to PK-C activation still requires investigation. Future studies directed at this problem will focus on the stimulation cascade of adherent cell activation and its physiologic control. In this context, elucidation of priming and deactivation promise to add another dimension to explaining physiologic parameters of neutrophil function.

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