Demonstration of Reversible Priming of Human Neutrophils Using Platelet-Activating Factor

By Elizabeth Kitchen, Adriano G. Rossi, Alison M. Condliffe, Christopher Haslett, and Edwin R. Chilvers

Exposure of neutrophils to agents such as lipopolysaccharide, tumor necrosis factor-α (TNF-α), and the granulocyte-macrophage colony-stimulating factor causes a major upregulation of subsequent agonist-induced NADPH oxidase activation. This priming effect is a prerequisite for neutrophil-mediated tissue damage and has been widely considered to be an irreversible process. We have investigated the potential for neutrophils to recover from a priming stimulus by studying the effects of platelet-activating factor (PAF). PAF did not stimulate respiratory burst activity directly, but caused a rapid (maximal at 10 minutes) and concentration-dependent (EC₅₀ 50.2 nmol/L) increase in N-formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated superoxide anion release. At time-points >10 minutes, this priming effect spontaneously declined, with return to basal, unstimulated levels by 120 minutes. An identical priming time-course was observed with N-methyl carbamyl PAF, a non-metabolizable analogue of PAF, indicating that the transient nature of PAF-induced priming was not secondary to PAF metabolism. Two structurally diverse PAF receptor antagonists (UK-74,505 and WEB 2066), added 10 minutes after PAF addition, increased the rate of decay of the priming effect. In contrast, TNF-α-induced priming, which was of a similar magnitude to that observed for PAF, was slower to evolve (maximal at 30 minutes) and remained constant for at least 120 minutes. The reversible nature of PAF-induced priming was confirmed by demonstrating that PAF-, but not TNF-α-, induced cell polarization (shape change) and CD11b-dependent neutrophil binding of albumin-coated latex beads was also transient, with return to basal, unstimulated levels by 120 minutes. Furthermore, cells that had spontaneously deprimed following PAF exposure retained their capacity to be fully reprimed by a subsequent addition of either PAF or TNF-α. These data imply that neutrophil priming is not an irreversible event: the demonstration of a cycle of complete priming, depriming, and repriming offers the potential for functional recycling of neutrophils at sites of inflammation.

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From the Respiratory Medicine Unit, Department of Medicine (RIE), University of Edinburgh Medical School, Rayne Laboratory, Edinburgh, United Kingdom.

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Address reprint requests to Edwin R. Chilvers, PhD, Respiratory Medicine Unit, Department of Medicine (RIE), University of Edinburgh Medical School, Rayne Laboratory, Teviot Place, Edinburgh EH8 9AG, UK.

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the PAF-primed superoxide anion response and PAF-induced CD11b/CD18 activation and to a lesser extent, PAF-induced shape change. The rate of neutrophil recovery following PAF addition could be enhanced by the use of selective PAF receptor antagonists (WEB 2086 and UK-74,505). Furthermore, the deprived cells retained their full capacity to be reprimed by an alternative priming agent (TNF-α) or a further addition of PAF. The ability of neutrophils to participate in a complete priming/depriming/repriming cycle allows far greater flexibility in the control of neutrophil behavior at an inflammatory site than was hitherto realized.

MATERIALS AND METHODS

Neutrophil preparation. Peripheral venous blood was taken from healthy adult volunteers, anticoagulated with 4 mL 3.8% sodium citrate/40 mL blood, and centrifuged (300g) for 20 minutes. Neutrophils were isolated exactly as previously detailed using dextran sedimentation and discontinuous Percoll gradients. This isolation technique yields neutrophils that display very low levels of basal shape change (<8% assessed flow-cytometrically) or direct fMLP-induced superoxide anion generation. The purified neutrophils were washed sequentially in platelet-poor plasma, phosphate-buffered saline (PBS) without, and PBS with, CaCl₂ and MgCl₂. Cell purity and viability (trypan blue exclusion) were routinely >95% (<0.5% monocyte contamination) and >99.5%, respectively.

Shape change assay. Neutrophils (10⁶ in 90 µL PBS containing CaCl₂ and MgCl₂) were equilibrated in a gently shaking water-bath for 5 minutes at 37°C. Priming agents were added in a 10-µL volume to achieve the required drug concentrations (PAF 1 nmoL/L to 10 µmoll/L, TNF-α 200 U/mL) and incubations continued for the periods stated. Preliminary experiments demonstrated this concentration of TNF-α to be optimal in causing maximal enhancement of fMLP-induced superoxide anion generation with minimal direct respiratory burst activation (data not shown). To determine the IC₅₀ values of the PAF receptor antagonists used, neutrophils were incubated with WEB 2086 or UK-74,505 (both at 10 nmoL/L to 10 µmoll/L) for 30 minutes before the addition of priming agents. For investigations examining the reversibility of PAF-induced priming, neutrophils were treated with PAF (1 µmoll/L) for 10 minutes before addition of 1 µmoll/L WEB 2086 or UK-74,505. fMLP (100 nmoLL) or buffer (PBS) was added to samples (final volume 1 mL) 10 minutes before the addition of an equal volume of 2.5% glutaraldehyde. Samples were analyzed for shape change by flow cytometry (Coulter EPICS Profile II, Coulter Electronics, Luton, UK) using a slight modification of a previously published method. Percentage shape change was calculated from the mean forward light scatter of each sample by gating on the non-shape changed neutrophil population. The values obtained using this method correlate closely with those derived by direct visual assessment of shape change, with the exception that the flow cytometric method of assessment slightly overestimates the extent of basal shape change.

Superoxide anion release assay. Neutrophils were isolated, equilibrated at 37°C, and incubated with PAF or PBS exactly as detailed above, except that cytochrome C (800 µL, 1 mg/mL) was added immediately before the addition of fMLP. One of each set of quadruplicate determinations included superoxide dismutase (375 U) to allow confirmation of the specificity of cytochrome C reduction. Reactions were stopped by placing the cells on ice, followed by centrifugation (12,500g 2 minutes, 4°C). The superoxide dismutase-inhibitable reduction of cytochrome C was determined in each supernatant by measuring the peak absorbance between 535 and 565 nm using a Pye-Unicam scanning spectrophotometer, and expressed as nanomoles superoxide anions generated per 10⁶ neutrophils. In experiments designed to assess the ability of PAF-recovered neutrophils to be reprimed with either TNF-α or a further addition of PAF, cells were treated with PAF (1 µmoll/L) or PBS for 120 minutes, before a final incubation with PAF (1 µmoll/L, 10 minutes) or TNF-α (200 U/mL, 30 minutes) and assessment of fMLP-stimulated superoxide anion release.

To examine the effects of hypotonic challenge on neutrophil function, cells (10⁶ in 250 µL PBS) were equilibrated at 37°C as outlined above and incubated for 19 minutes in PBS containing 80 µmoll/L cytochrome C, with 150 mmoll/L NaCl (isotonic incubations) or 50 mmoll/L NaCl (hypotonic incubations). Neutrophils were then treated for 1 minute with 20 µL of either 5 mol/L NaCl (to reverse hypotonicity to isotonicity) or PBS (to retain hypotonicity or isotonicity). The effects of the hypotonic challenge itself on basal and fMLP-stimulated superoxide anion generation, together with the ability of these cells to deprime following restoration of isotonicity and thereafter reprimed with PAF, was then assessed as detailed above.

Neutrophil adhesion to albumin-coated latex beads (ACLB). Fluorescent latex beads (2.5% packed vol/vol stock solution as purchased) were washed (three times) in PBS, resuspended at 2.5% (vol/vol) in PBS containing 10 mg/mL human serum albumin, and incubated for 19 minutes at 25°C. The resultant ACLB were then washed (three times) in PBS and finally resuspended at 0.75% (vol/vol). Neutrophils (175 µL aliquots at 10⁷/mL, in PBS with CaCl₂ and MgCl₂) were incubated in a shaking water-bath at 37°C for 5 minutes, and then treated with PAF (1 µmoll/L), TNF-α (200 U/mL) or PBS (all added in a 1.5-µL volume) for 0 to 120 minutes. ACLB (25 µL of 0.75% vol/vol solution) were added to each tube 15 minutes before the termination of each reaction, except for time points <15 minutes where the beads were added before the agonist. Neutrophils were then fixed by the addition of 0.5 mL of 0.5% glutaraldehyde. After 30 minutes at room temperature, sonohardened ACLB were removed by washing with PBS (three times) and bead-binding to the neutrophils assessed using an EPICS Profile II (Coulter Electronics), as previously detailed.

Statistics. All values are expressed as means ± standard error of the mean (SEM) (n) number of separate experiments. Values, where applicable, were compared by ANOVA or the Student’s t-test for paired data, with P < .05 considered to be significant. Significant differences between groups were determined by the Newman-Keuls procedure.

Materials. fMLP, PAF, superoxide dismutase (SOD), cytochrome C, PBS (with or without CaCl₂ and MgCl₂), dextran-500, Percoll, human serum albumin and glutaraldehyde (25%) were purchased from Sigma (Poole, UK). TNF-α was obtained from Genzyme (Cambridge, MA). One micron fluorescent microspheres were purchased as a 2.5% solids-latex (2.5% vol/vol) stock solution from Polysciences Inc through the UK supplier Park Scientific (Nottingham, UK). WEB 2086 and UK-74,505 was gifts from Boehringer Ingelheim Ltd (Berk, UK) and Dr I Parry (Pfizer, Sandwich, UK), respectively. 1-O-alkyl-2-N-methyl carbamyl-glycerophosphocholine (N-methyl carbamyl PAF) was obtained from Calbiochem (Nottingham, UK).

RESULTS

PAF is an established and important inflammatory mediator in vivo. It was selected for this study because its priming effect in neutrophils is rapid, receptor-mediated, associated with minimal direct activation of superoxide anion release, and inhibitable by specific, high-affinity PAF receptor antagonists. Under our own experimental conditions, PAF (1 nmoll/L to 10 µmoll/L) did not affect spontaneous superoxide anion release (Fig 1A), but caused a rapid (maximal at 10 minutes), concentration-dependent increase in fMLP-stimulated superoxide anion release (EC₅₀ 50.2 nmoll/L).
A concentration-dependent increase in shape change (EC₅₀ 110 nmol/L, Fig 1B). In the shape change experiments, 10 μmol/L PAF appeared to have less effect than 1 μmol/L PAF, but this correlated with the light-microscopic observation of large, round, "glassy"-looking cells, suggestive of cell swelling. In view of these findings, a PAF concentration of 1 μmol/L was chosen for all further priming studies.

PAF-induced priming of superoxide anion generation in neutrophils is reversible. Figure 2 illustrates how the length of the initial PAF incubation period affects the subsequent enhancement of superoxide anion release in response to fMLP. The ability of PAF to prime the fMLP-response was maximal after a 10-minute PAF preincubation, with the priming effect decaying thereafter, to approach unprimed levels by 2 hours (Fig 2). Notably, the pattern of reversal of the PAF priming effect was consistently biphasic, with an initial rapid loss in priming occurring within 15 to 30 minutes of PAF addition, followed by a second, slower phase of decay. This progressive decline in the magnitude of the primed fMLP-superoxide anion response was not due to cell necrosis, as viability (assessed by trypan blue exclusion) was routinely >95% for all time points studied.

Fig 1. Effect of PAF on basal and fMLP-stimulated superoxide anion generation and shape change in human neutrophils. (A) Concentration-response data for superoxide anion generation. Isolated human neutrophils (10⁶ in 90 μL PBS) were equilibrated for 5 minutes at 37°C and incubated with 10 μL of PAF (10 nmol/L to 10 μmol/L final concentration) for 10 minutes. Cells were then stimulated with 100 nmol/L fMLP (hatched bars) or buffer (closed bars) for 10 minutes in the presence of cytochrome C (1 mg/mL), in a final volume of 1 mL. Reactions were terminated by placing samples on ice and superoxide anion release was assessed by scanning spectrophotometry. Values represent mean of triplicate determinations from a single experiment, representative of six. (B) Concentration-response data for shape change. Neutrophils were incubated as outlined above for superoxide anion generation, except that buffer replaced the cytochrome C and reactions were terminated by the addition of 1 mL 2.5% glutaraldehyde. Samples were analyzed by flow cytometry and percent shape change calculated from the mean forward light scatter values, by gating on the non-shape changed population. Values represent mean ± SEM for six independent experiments each performed in duplicate.

Fig 2. Time-course for PAF-priming of fMLP-stimulated superoxide anion release in human neutrophils. Isolated human neutrophils (10⁶ in 80 μL PBS) were equilibrated for 5 minutes at 37°C and incubated with 10 μL PAF (1 μmol/L) (closed symbols) or buffer (open symbols) for 10 minutes. A 10-μL aliquot of WEB 2086 (1 μmol/L, triangles), UK-74,505 (1 μmol/L, diamonds), or buffer (circles) was added 10 minutes after PAF. Cells were incubated for a further 0 to 120 minutes before a final 10-minute stimulation with 100 nmol/L fMLP (circles, triangles, diamonds) or buffer (squares) in the presence of cytochrome C (1 mg/mL). Reactions were terminated at the appropriate times by placing the cells on ice and superoxide anion release assessed by scanning spectrophotometry. Data points represent mean values for triplicate determinations from three separate experiments. SEM values were all <10 % of mean and are omitted for reasons of clarity.
In an attempt to elucidate some of the possible factors responsible for this time-dependent reversal of PAF-mediated neutrophil priming, additional experiments were undertaken to assess the role of PAF metabolism and PAF receptor desensitization. Firstly, PAF was substituted by a nonmetabolizable analogue, N-methyl carbamyl PAF. This resulted in a near identical time course (data not shown) to that illustrated in Fig 2, indicating that PAF degradation was not responsible for the loss in the priming effect. Secondly, we examined the ability of two specific, but structurally different, PAF receptor antagonists, WEB 2086 and UK-74,505, to influence the rate of depriming. Preliminary studies established optimal conditions for the use of these antagonists: a 30-minute preincubation with UK-74,505 caused a concentration-dependent (IC50, 68 nmol/L) inhibition of the PAF-primed superoxide anion response whereas inhibition by WEB 2086 was biphasic and incomplete (55% ± 4% inhibition with 10 μmol/L WEB 2086, data not shown). Neither of these compounds, at the concentrations used, affected neutrophil viability nor superoxide anion release in control or fMLP-treated cells (data not shown). Thus, to investigate the influence of PAF receptor blockade on the rate of decay of PAF-induced priming, both antagonists were used at a concentration of 1 μmol/L. When WEB 2086 (1 μmol/L) or UK-74,505 (1 μmol/L) was added 10 minutes after PAF, a small but significantly faster (P < .05) rate of decay of the PAF-primed superoxide anion response was observed (Fig 2), with UK-74,505 having the greater effect. This data suggests that although PAF receptor desensitization or uncoupling may play a role in the decay of the priming effect, this process is not complete following a 10-minute incubation with 1 μmol/L PAF.

In marked contrast to the priming time course observed with PAF, TNF-α-induced priming, although slower to evolve (maximal at 30 minutes), remained constant for at least 2 hours (nanomoles superoxide anion released: at 30 minutes: fMLP (100 nmoVL) 4.6 ± 1.6, TNF-α (200 U/mL) + fMLP 20.1 ± 3.2; at 2 hours: fMLP 5.0 ± 0.7, TNF-α + fMLP 19.2 ± 4.9, n = 3).

**Transient effects of PAF on neutrophil shape change and ACLB binding.** To validate our observations of neutrophil recovery following PAF-mediated priming of the respiratory burst, we undertook further time course studies to examine the effects of PAF on neutrophil shape change and CD11b activation. Shape change was chosen because previous studies have demonstrated a tight correlation between priming of the superoxide anion response and the extent or proportion of neutrophils that have undergone cell polarization. In addition, the relatively weak priming effect of IL-8 on superoxide anion generation has been reported to partially reverse with time without any concomitant recovery of CD11b expression.

Incubation of neutrophils with 1 μmol/L PAF caused a rapid (maximal at 2 minutes) increase in shape change, which then declined spontaneously towards basal levels by 30 minutes (Fig 3). The subsequent small increase in percent shape change, observed between 30 to 120 minutes, paralleled the changes seen in control cells. The extent of the initial shape change response to PAF was similar to that observed following a 10-minute incubation with 100 mmol/L fMLP (80.8% ± 7.1%, n = 3). It should be noted here that flow-cytometric quantification...
of shape change, although a convenient and highly reproducible assay, gives consistently higher levels of basal shape change (approximately 8% \(^1\)) than those obtained by direct visual assessment of cell morphology. Our data confirm, however, that PAF-induced shape change, like priming of the superoxide anion response, is a transient event. Neutrophil shape change to TNF-\(\alpha\) (200 U/mL) was of a similar magnitude (70.1% \(\pm\) 1.8%, \(n=4\)) to that induced by PAF, but was slower to evolve (plateau at 30 minutes) and remained constant for the 2-hour incubation period (Fig 3).

As a third index of priming, we followed the \(\beta_2\)-integrin-dependent binding of ACLB over a 2-hour incubation with either PAF or TNF-\(\alpha\). Again, PAF (1 \(\mu\)mol/L) induced a time-dependent increase in ACLB binding, maximal after 10 minutes, which declined to reach control levels by 2 hours (Fig 4). TNF-\(\alpha\) (200 U/mL) also augmented ACLB binding but, unlike PAF, the extent of bead binding reached a plateau at 30 minutes and remained constant for the ensuing 90-minute incubation period (Fig 4).

**Fig 4.** Time-course for PAF- and TNF-\(\alpha\)-induced binding of ACLB. (a) Representative flow-cytometry histograms of control cells (A) and cells incubated with PAF for 10 minutes (B) or 120 minutes (C) (x-axis shows logarithmic scale green fluorescence (LFL) and y-axis shows relative cell number). The lowest fluorescent (far left) peak represents neutrophils with no attached ACLB then, with increasing binding, single, double, and triple bead peaks (correlated by fluorescence microscopy) can be distinguished. The percent neutrophils with attached ACLB was calculated by gating out the far left peak determined from time-matched control samples. (b) Time-course data for PAF- and TNF-\(\alpha\)-induced binding of ACLB. Isolated human neutrophils (1.75 \(\times\) 10\(^6\) in 175 \(\mu\)L PBS) were equilibrated for 5 minutes at 37\(^\circ\)C and incubated with 15 \(\mu\)L PAF (1 \(\mu\)mol/L) (closed circles), TNF-\(\alpha\) (200 U/mL) (closed squares), or buffer (open squares) for 0 to 120 minutes. ACLB (25 \(\mu\)L) were added 15 minutes before termination of the reaction with 0.5 mL 0.5% glutaraldehyde, except for time points <15 minutes where the beads were added before the agonist. Samples were analyzed by flow cytometry and the percent neutrophils with attached ACLB was calculated, as detailed above. Values represent mean \(\pm\) SEM for four independent sets of determinations, each performed in duplicate. Where not shown, SEM values fall within the symbols.

Repriming of neutrophils with PAF or TNF-\(\alpha\). We next investigated whether neutrophils that had primed and then spontaneously deprimed during a 120-minute incubation with PAF were capable of being reprimed. Figure 5 demonstrates that PAF-recovered neutrophils retain their full capacity to be reprimed when challenged again with either PAF (1 \(\mu\)mol/L, 10 minutes) or TNF-\(\alpha\) (200 U/mL, 30 minutes), generating similar amounts of superoxide anions upon fMLP stimulation as freshly-primed cells. This finding was repeated when neutrophils were primed and deprimed using a modification of a previously described hypotonic challenge protocol.\(^2\) Under isotonic conditions, fMLP (100 nmol/L) and PAF (1 \(\mu\)mol/L) alone elicited little superoxide anion release, with PAF enhancing the fMLP-stimulated superoxide anion response by 3.8-fold (Table 1). A 20-minute hypotonic challenge resulted in a modest (twofold) priming of the fMLP response, which recovered towards control levels when isotonicity was restored for 1 minute. Subsequent treatment with PAF reprimed the fMLP response, albeit to a slightly lower level than that observed in cells maintained under isotonic conditions. Cell viability was routinely \(>95\%\) for all conditions studied. Thus, like cells that had deprimed following PAF exposure, osmotically primed and deprimed neutrophils also retained their capacity to be primed for a second time by a physiological agonist such as PAF.

**DISCUSSION**

The neutrophil can exist in a number of different functional states and this has a significant bearing on its behavior...
and responsiveness in vitro. Thus, in the unprimed state, the neutrophil displays little or no secretory response when incubated with an agent such as fMLP, whereas such a challenge in a fully primed cell results in an explosive increase in respiratory burst activity; this priming-activation axis has been shown to be a major determinant of neutrophil behavior in vivo. However, the very protracted priming effect of agents such as LPS, G-CSF, and GM-CSF, together with the short life-span of the neutrophil, has led to the belief that priming is a largely irreversible process. Indeed, the sustained nature of the priming effect has been postulated to play a fundamental role in the long-term inflammatory response observed with certain agents, including endotoxin.

In this report, we provide evidence that neutrophil priming is not an irreversible process and, moreover, that these cells, once deprived, can go through a further complete cycle of priming and activation.

The depriming of neutrophils observed following PAF treatment was apparent for fMLP-stimulated superoxide anion generation, CD11b function, and cell polarization, and hence was unlikely to represent selective downregulation of one particular component of the priming response, as reported with IL-8. The ability of neutrophils to be reprimed by TNF-α and PAF after a 2-hour incubation, with maintenance of full viability throughout, excludes the possibility that the loss of the PAF priming effect was merely a consequence of the extended incubation procedure affecting cell integrity or metabolic status. While the basis for the decline in PAF-mediated priming is uncertain, the identical nature of the time-course of priming of superoxide anion release with N-methyl carbamyl PAF, a biologically active PAF analog that is completely resistant to metabolic inactivation by neutrophils or human serum, makes PAF metabolism unlikely. We have also shown that inclusion of adenosine deaminase in these incubations does not influence the time-
course of PAF-primed superoxide anion responses, which
excludes a secondary effect mediated via adenosine release
and autocrine activation of cyclic adenosine monophosphate
(AMP)-dependent protein kinase pathways (Kitchen, Rossi
and Chilvers, unpublished observations, February 1995). Al-
though homologous receptor desensitization may underlie
the transient nature of the PAF signal, the increased rate of
decay of fMLP-induced superoxide anion release following
UK-74,505 or WEB 2086 addition and the return of a fully
competent PAF priming response after 2 hours suggests that
PAF receptor uncoupling/desensitization is both incomplete
and transient. These data are consistent with previous studies
demonstrating rapid activation-induced uncoupling and in-
ternalization of PAF receptors followed by subsequent recep-
tor re-expression. The recovery of PAF receptor number and
function is likely to reflect extensive membrane attach-
ment and metabolism of PAF (approximately 1 pmol/10^7
neutrophils/min).24,25

From our own comparisons of PAF and TNF-α and other
published observations, it would appear that neutrophil prim-
ing in vitro falls into three categories: (1) fully reversible
(eg, that induced by PAF, osmotic swelling, or inositol hexa-
kisphosphate); (2) partially reversible (eg, with IL-1 or IL-
8); or (3) largely irreversible (eg, with GM-CSF, G-CSF,
or LPS). Further studies would be required to categorize
TNF-α because its effects did not show any signs of recovery
over the 2-hour incubation period used in this study, but have
been reported to decay over 24 hours.7 It is also intriguing to
note that only agents in group (3) are able to modulate the
rate of neutrophil apoptosis, which again testifies to the long
duration of action of this class of agents.26 While it is clear
that neither the efficacy nor extent of the initial priming
signal dictates the reversibility of the primed state (because
PAF, TNF-α [Fig 5], LPS, and GM-CSF [data not shown]
induce equivalent levels of priming), it is possible that the
duration of the priming signal and/or its rate of onset are
key determinants. However, in the absence of any clear
mechanistic basis for neutrophil priming, it is also possible
that the above agents use discrete signalling pathways to
induce their priming effects.

This current observation of reversible priming may allow
the pro-inflammatory, and potentially tissue-damaging, ef-
fects of neutrophil priming/activation to be counteracted by
a process other than apoptosis or the pharmacological inhibi-
tion of neutrophil activation. It is unlikely that neutrophils
within an inflammatory focus would be exposed in isolation
to PAF or other “transient” priming agents. However, as
endothelial cell-association PAF has been shown to play a
central role in neutrophil priming and migration through IL-
1β—treated human umbilical vein endothelial cell mono-
layers in vitro,27 and endogenously formed PAF is involved
in leukocyte extravasation induced by IL-1 in vivo,28 any
delay in cell exit through an activated endothelial surface
may permit cell recovery and the return of unprimed neutro-
phils to the circulation. Thus, the recognition that neutrophils
have the potential to deprime allows an additional point of
control in the early stages of the acute inflammatory re-
sponse, whereby cells may return to their former quiescent
state and potentially re-enter the circulating neutrophil pool.
These deprimed neutrophils, once fully recovered, could
again attain their maximal priming potential and mount sub-
sequent responses, as dictated by ensuing inflammatory chal-
lenges. In contrast, priming agents with a longer duration
of action would maintain neutrophils in the primed state for
a much longer period of time and may, therefore, play a dis-
tinct role in vivo, in the wake of a more widespread or
prolonged inflammatory insult.

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