Stimulation of Intracellular Ca\textsuperscript{2+} Levels in Human Neutrophils by Soluble Immune Complexes

FUNCTIONAL ACTIVATION OF FcγRIIIb DURING PRIMING

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Soluble immune complexes bind to unprimed neutrophils and generate intracellular Ca\textsuperscript{2+} transients but fail to activate the NADPH oxidase. Following priming of the neutrophils with either tumor necrosis factor α or granulocyte-macrophage colony-stimulating factor, stimulation of the cells with the soluble immune complexes leads to an enhanced Ca\textsuperscript{2+} signal and significant secretion of reactive oxidants. The enhanced Ca\textsuperscript{2+} signal observed in primed neutrophils results from the influx of Ca\textsuperscript{2+} from the external environment and is partly sensitive to tyrosine kinase inhibitors. This is in contrast to the Ca\textsuperscript{2+} signal observed in unprimed neutrophils, which arises from the mobilization of intracellular stores. When the surface expression of FcγRIIIb on primed neutrophils was decreased either through incubation with Pronase or phosphoinositide-specific phospholipase C, the extra enhanced Ca\textsuperscript{2+} mobilization seen in primed cells was significantly lowered, while the initial rise in intracellular Ca\textsuperscript{2+} was unaffected. Depletion of FcγRIIIb had no significant effect on the Ca\textsuperscript{2+} transients in unprimed neutrophils. Cross-linking FcγRII, but not FcγRIIIb, induced increases in intracellular Ca\textsuperscript{2+} in unprimed neutrophils, while cross-linking either of these receptors increased Ca\textsuperscript{2+} levels in primed neutrophils. The FcγRII-dependent intracellular Ca\textsuperscript{2+} rise in primed cells was unaffected by incubation in Ca\textsuperscript{2+}-free medium, whereas the FcγRIIIb-dependent transient was significantly decreased when Ca\textsuperscript{2+} influx was prevented in Ca\textsuperscript{2+}-free medium supplemented with EGTA. Cross-linking either FcγRII or FcγRIIIb in primed or unprimed cells failed to stimulate substantial levels of inositol 1,4,5-trisphosphate production. These results indicate that following stimulation of primed neutrophils with soluble immune complexes the enhanced Ca\textsuperscript{2+} mobilization observed is the result of a functional activation of the glycosylphosphatidylinositol-linked FcγRIIIb.

Neutrophils play a major role in host defense via the phagocytosis and destruction of pathogens during acute inflammation. The binding of opsonized bacteria or immune complexes to neutrophil immunoglobulin receptors (FcγR) can activate a number of processes such as phagocytosis, degranulation and activation of the NADPH oxidase (1, 2). However, in addition to this protective role, inappropriate activation of neutrophils to release oxygen metabolites and granule enzymes within tissues can result in tissue damage in inflammatory conditions such as rheumatoid arthritis (1, 2). Furthermore, immune complex deposition followed by subsequent neutrophil activation is important in the pathogenesis of serum sickness, the Arthus reaction, acute glomerulonephritis, and other idiopathic inflammatory diseases (3, 4). Neutrophils are activated within the synovial joints of rheumatoid patients (5–10), and we have shown that synovial fluid from patients with rheumatoid arthritis contains both soluble and insoluble IgG-containing immune complexes, which are capable of activating neutrophils (11–13). However, the soluble immune complexes only activate neutrophils that have been previously primed in vivo or in vitro by GM-CSF or γ interferon and this activation leads to the secretion of reactive oxidants and granule enzymes (11–13). In contrast, insoluble immune complexes activate both primed and unprimed neutrophils with nearly equal efficacy but the oxidants generated are largely retained intracellularly, i.e. within phagolysosomes. Thus, soluble immune complexes may be of greater importance than insoluble complexes in the destructive processes that occur during inflammatory diseases because they activate the secretion of large quantities of cytotoxic products. The identification of the molecular processes that occur during neutrophil priming to allow activation of oxidant secretion in response to soluble complexes may therefore be of value in identifying new targets for therapeutic intervention.

IgG-containing immune complexes stimulate neutrophils via Fcγ receptors, two types of which are expressed on freshly isolated control blood cells (14). FcγRI (CD32) is a 40-kDa transmembrane-spanning molecule with a cytoplasmic tail that allows its interaction with G-proteins, whereas FcγRIIIB is a heavily glycosylated molecule of 50–70 kDa, linked to the membrane via a glycosylphosphatidylinositol anchor (15). Thus, although FcγRIIIB has the potential for rapid lateral mobility within the membrane, it is unable to couple directly to either G-proteins or to the cytoskeleton, and it is unclear how ligation to this receptor might lead to the transduction of intracellular signals. Expression of FcγRIIIB on the plasma membrane (100,000–200,000 molecules/cell) is approximately 10–15-fold greater than the expression of FcγRII (7000–15,000 molecules/cell).

There is much debate as to the independent and co-operative roles of FcγRII and FcγRIIIb in neutrophil activation. Neither receptor binds monomeric IgG, but they bind dimers, trimers, immune complexes, and opsonized particles (16, 17). Although

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1 The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; mAb, monoclonal antibody; PI, phosphoinositide; PLC, phospholipase C; TNFα, tumor necrosis factor α; IP\textsubscript{3}, inositol 1,4,5-trisphosphate.
Neutrophil Activation by Soluble Immune Complexes

In this study, we have investigated Ca\(^{2+}\) mobilization in neutrophils stimulated with soluble immune complexes in both primed and unprimed neutrophils. We present evidence that the initial cytosolic Ca\(^{2+}\) increase observed following stimulation with soluble complexes is the result of ligation of FcRII and that this interaction is insufficient to activate the respiratory burst. When soluble complexes are added to primed neutrophils, activation secretion is initiated and an enhanced mobilization of Ca\(^{2+}\) results, mainly via influx from extracellular sources. It would appear that this extra intracellular Ca\(^{2+}\) signal results from functional activation of FcRII in priming these studies add new insights into the mechanisms by which cellular priming alters the functional responsiveness of neutrophils during inflammatory activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Neutrophil isolation medium was from Cell Applications Inc., RPMI 1640 medium from Flow Laboratories; Fluo-3-AM, National Institute for Biological Standards and Controls, Potters Bar, United Kingdom. Fab/F(ab\(^{\prime}\))\(_2\) fragments of appropriate monoclonal antibodies at 37 °C prior to cross-linking with 40 μg/ml F(ab\(^{\prime}\))\(_2\) fragments of goat anti-mouse IgG. For mAb blocking studies, 3 μg of Fab/F(ab\(^{\prime}\))\(_2\) fragments in a volume of 250 μl for 10 min at 37 °C prior to dilution (to 5 x 10\(^5\) cells/ml) and stimulation of cells with soluble immune complexes.

**Insolositol 1,4,5-Trisphosphate Assays**—Neutrophils were incubated for 45 min in the presence (primed) and absence (control) of 50 units/ml GM-CSF. FcRII and FcRIIIb were then cross-linked (as described above), and samples were removed at time intervals. IP\(_3\) was then extracted by rapidly mixing samples with 0.2 volume of ice-cold 20% (v/v) perchloric acid. After neutralizing extracts, IP\(_3\) was quantified using a competitive binding assay (Amersham, UK) using m-tyrosyl-inositol 1,4,5-trisphosphate as a calibration standard.

**RESULTS**

**Reactive Oxidant Generation Induced by Soluble Immune Complexes**—Addition of synthetic soluble IgG immune complexes to freshly isolated neutrophils failed to activate reactive oxidant generation (Fig. 1), as detected by Luminol chemiluminescence. However, if the cells were primed by prior addition of GM-CSF (50 units/ml for 1 h) or TNFα (50 ng/ml for 10 min), then the soluble complexes activated a rapid and transient burst of oxidative activity that peaked 2–3 min after addition (Fig. 1, A and C). Luminol-dependent chemiluminescence detects both intracellular and extracellular oxidant production (39), but the use of cytochrome c, which is a large basic molecule that cannot permeate the cell, specifically measures O\(_2\)\(^{\bullet-}\) secretion (38). The addition of soluble immune complexes to unprimed cells similarly did not activate O\(_2\)\(^{\bullet-}\) secretion but did lead to a rapid and significant secretion of O\(_2\)\(^{\bullet-}\) that had been either primed with TNFα or with GM-CSF (Fig. 1, B and D). Intracellular Ca\(^{2+}\) Mobilization—Resting levels of intracellular Ca\(^{2+}\) were approximately 140 nM (n = 18) in unstimulated neutrophils. When soluble immune complexes were added to unprimed cells, there was a rapid and transient monophasic increase in the level of intracellular Ca\(^{2+}\), which reached a maximal level of 0.9–1.0 μM Ca\(^{2+}\) approximately 30–45 s following stimulation (Fig. 2). This increase in intracellular Ca\(^{2+}\) was comparable to that induced by addition of 0.1 μM fMet-Leu-Phe (data not shown). When the neutrophils were primed by exposure to TNFα prior to stimulation with the soluble complexes, there was again a rapid rise in intracellular Ca\(^{2+}\) levels (Fig. 2A), but the kinetics of the increase were...
altered. The initial increase in intracellular Ca\(^{2+}\) seen in unprimed neutrophils was unaffected, but this was followed by a second, more sustained increase in intracellular Ca\(^{2+}\). Similarly, when the neutrophils were primed with GM-CSF (Fig. 2B), the intracellular Ca\(^{2+}\) mobilization induced by the soluble immune complexes was enhanced and prolonged in comparison to that observed in unprimed cells. Increasing the concentrations of soluble immune complexes (to 20% and 30%, v/v) did not change either the kinetics or the magnitude of the intracellular Ca\(^{2+}\) transients seen in unprimed cells. Thus, priming does not affect the affinity of binding of soluble immune complexes under these conditions.

**Effect of EGTA on Intracellular Ca\(^{2+}\) Mobilization**—To determine the source of the increased intracellular Ca\(^{2+}\) levels in both unprimed and primed neutrophils, incubations in Ca\(^{2+}\)-free medium in the presence of 1 mM EGTA were performed. Fig. 3A shows that when unprimed cells were stimulated with soluble immune complexes in Ca\(^{2+}\)-free buffer, there was no significant difference in the kinetics of intracellular Ca\(^{2+}\) changes compared with those obtained in Ca\(^{2+}\)-containing buffer. Thus, in unprimed neutrophils, the observed increases in intracellular Ca\(^{2+}\) arise from the mobilization of intracellular Ca\(^{2+}\) stores. In contrast, when primed cells were stimulated with soluble immune complexes Ca\(^{2+}\)-free buffer, the initial intracellular Ca\(^{2+}\) increase was unaffected, but the extended “extra” Ca\(^{2+}\) signal only seen in primed cells, was not observed (Fig. 3, B and C). These experiments thus indicate that the enhanced and prolonged increase in intracellular Ca\(^{2+}\) observed only in primed cells is due to Ca\(^{2+}\) influx from external sources.

**Role of Tyrosine Kinases in Ca\(^{2+}\) Mobilization**—When unprimed neutrophils were incubated with the tyrosine kinase inhibitor, erstatin (1 μg/ml), the intracellular Ca\(^{2+}\) response...
obtained following stimulation with the soluble complexes was inhibited by 30% (± 8%, n = 6). When neutrophils were primed with either TNFα (Fig. 4B) or GM-CSF (Fig. 4C), the enhanced intracellular Ca\(^{2+}\) signal seen only in primed neutrophils was similarly decreased. This concentration of erstatin decreased reactive oxidant generation stimulated by soluble immune complexes in primed neutrophils by >95% (data not shown). Similar results were obtained following addition of another tyrosine kinase inhibitor genistein at 100 μM (data not shown). Addition of erstatin at 2 μg/ml inhibited the unprimed intracellular Ca\(^{2+}\) signal by over 50%, but did not result in greater inhibition of the primed response (data not shown).

To determine whether erstatin exhibited nonspecific toxic effects at these concentrations, the experiments shown in Fig. 5 were performed. After addition of soluble immune complexes to primed neutrophils incubated in the presence of erstatin, fMet-Leu-Phe was added. The intracellular Ca\(^{2+}\) transients stimulated by fMet-Leu-Phe were unaffected by the inhibitor; this fMet-Leu-Phe-induced response was identical to that observed following its addition to suspensions that had not previously been stimulated by immune complexes.

**Role of Fcγ Receptors: Depletion of Surface Receptors**—When neutrophils were incubated with PI-PLC (at 0.25 units/ml for 30 min), the surface expression of FcγRIIib, which is glycosylphosphatidylinositol-linked (14, 15), detected by fluorescent-activated cell sorting was decreased to levels that were indistinguishable from nonspecific antibody binding (Fig. 6A, i). This treatment had no effect on the expression of FcγRII (Fig. 6A, ii) or on the expression of CD11b (data not shown). Similarly, incubation with Pronase (0.05 mg/ml for 30 min) also resulted in the decreased surface expression of FcγRIIib but had no effect on expression of FcγRII or CD11b (data not shown).

When FcγRIIib expression on the surface of primed neutrophils was depleted by treatment with either Pronase or PI-PLC, the soluble immune complexes failed to activate the respiratory burst (Fig. 6, inset). Neutrophils treated in this way can, however, still generate reactive oxidants in response to large, insoluble immune complexes or PMA (13).

When unprimed neutrophils were depleted of surface FcγRIIib by treatment with PI-PLC, there was very little effect on the intracellular Ca\(^{2+}\) rise stimulated by soluble immune complexes (Fig. 6B, i). This result indicates that expression of FcγRIIib is not required for the transient, intracellular Ca\(^{2+}\) increase that arises from the mobilization of intracellular stores following the binding of soluble immune complexes to the surface of unprimed cells. However, in primed cells, Pronase treatment had a marked effect on the intracellular Ca\(^{2+}\) signals (Fig. 6B, ii). In primed cells depleted of FcγRIIib, the initial intracellular Ca\(^{2+}\) transient was unaffected, but the later, sustained intracellular Ca\(^{2+}\) signal that arises from Ca\(^{2+}\) influx was not observed. Similar results were obtained when the neutrophils were primed with TNFα and when FcγRIIib was depleted from the cell surface by incubation with Pronase (data not shown). These data thus indicate distinct roles for individual FcγR in the control of these intracellular Ca\(^{2+}\) signals in primed and unprimed neutrophils. Thus, the initial intracellular Ca\(^{2+}\) transient that is seen in both unprimed and primed neutrophils appears to be due to signals generated via occupancy of FcγRII; this intracellular Ca\(^{2+}\) signal arises from the mobilization of intracellular stores. In contrast, the extra intracellular Ca\(^{2+}\) signal that is only seen in primed cells requires FcγRIIb and arises from Ca\(^{2+}\) influx.

**Role of FcγR Ligation of Individual Receptors**—To confirm the above conclusions on the roles of FcγRI and FcγRIIib in the differential intracellular Ca\(^{2+}\) signals, cross-linking Fab/F(ab\(^\prime\))\(_2\) fragments of specific anti-FcγR mAbs was employed. Incubation of unprimed neutrophils with Fab fragments of IV3 (anti-FcγRII), followed by cross-linking with goat anti-mouse F(ab\(^\prime\))\(_2\)
Neutrophil Activation by Soluble Immune Complexes

In this study we have investigated the mechanisms by which soluble immune complexes activate neutrophils via Fcγ receptors. Defining this interaction is important because several immune conditions are associated with neutrophil activation via immune complexes (1-3). These soluble immune complexes fail to activate the respiratory burst when added to suspensions of unprimed cells. However, if the cells are primed (as in our study using the cytokines GM-CSF and TNFα), then soluble immune complex binding results in the secretion of large quantities of reactive oxygen metabolites. Because neutrophil function in vivo is invariably regulated by exposure to priming agents and because these soluble immune complexes activate the secretion of potentially tissue-damaging components, these results are important in understanding the molecular pathology of certain neutrophil-mediated conditions. In our study, we have used a neutrophil isolation procedure that induces minimal priming during cell isolation. Many commonly used isolation methods (33, 34, 42), however, inadvertently prime both receptor number/function and NADPH oxidase activity and hence experiments with cells isolated by such methods cannot clearly distinguish between neutrophil responses that are restricted to either the primed or the unprimed state.

Whereas the soluble immune complexes failed to activate the NADPH oxidase in unprimed cells, the use of fluorescein isothiocyanate-labeled complexes showed that they clearly bind to the cell surface (data not shown). These fluorescein isothiocyanate-labeled complexes activated neutrophils in identical ways to the unlabeled complexes, indicating that their molecular properties were unaltered during the labeling process. We have previously shown that the synthetic soluble immune complexes used in these studies and those isolated from rheumatoid synovial fluid, activate primed and unprimed neutrophils by analogous mechanisms (12, 43). The fact that these complexes activated increases in intracellular Ca²⁺ in unprimed cells is indicative that they bind to functionally active receptors on the cell surface. Hence, we conclude that the key event that occurs during priming that allows the primed cell to generate reactive oxidants in response to soluble immune complexes is not increased complex binding. Indeed, priming results in only small changes in the level of expression of either FcγRI or FcγRIIb, whereas surface expression of CR3 is greatly up-regulated (44). We thus searched for events downstream of receptor/ligand binding that may account for the altered functional responses of primed cells.

In unprimed cells, the soluble immune complexes activated a transient increase in intracellular Ca²⁺ that was due to mobilization of intracellular stores and was partly sensitive to inhibition with tyrosine kinase inhibitors. Several other reports have shown that, while intracellular Ca²⁺ transients are necessary for NADPH oxidase activation, they are in themselves not sufficient to induce activation (45, 46). When the cells were primed, however, an extra intracellular Ca²⁺ transient was...
generated. This arose from influx of $\text{Ca}^{2+}$ from external sources and was also partly sensitive to inhibition by tyrosine kinase inhibitors. These data indicate that this extra intracellular $\text{Ca}^{2+}$ signal may be due to signals generated via receptors that become "activated" during the priming process. We thus set out to identify these putative receptors.

Circulating blood neutrophils possess two types of receptors that recognize IgG-containing immune complexes, namely $\text{Fc}_{\gamma}RII$ and $\text{Fc}_{\gamma}RIIIb$ (14). $\text{Fc}_{\gamma}RIIIb$ is linked to the plasma membrane via a glycosylphosphatidylinositol linkage that can...
Ca²⁺. Fluo-3-loaded neutrophils were primed and then incubated as described in the legend to Fig. 7. Suspensions were incubated either in Ca²⁺-free medium (supplemented with 1 mM EGTA) or Ca²⁺-containing medium (1 mM), prior to cross-linking FcγRs.

FIG. 8. Role of extracellular Ca²⁺ in FcγRII mediated intracellular Ca²⁺ transients. Fluo-3-loaded neutrophils were primed and then incubated as described in the legend to Fig. 7. Suspensions were incubated either in Ca²⁺-free medium (supplemented with 1 mM EGTA) or Ca²⁺-containing medium (1 mM), prior to cross-linking FcγRs.

be easily cleaved either in vivo or in vitro with Pronase or PI-PLC (15, 17). Our first experiments thus used Pronase and PI-PLC to deplete the surface of FcγRIIb while not affecting expression of FcγRII. FcγRIIb-depleted (unprimed) neutrophils could generate intracellular Ca²⁺ transients in response to soluble immune complexes, and the kinetics of these transients were identical with those observed in untreated (FcγRIIb-expressing) cells. However, the extra intracellular Ca²⁺ signal normally observed during priming was not observed after FcγRIIb depletion. Furthermore, this extra intracellular Ca²⁺ signal was not observed when primed cells were incubated F(ab)₂ fragments of 3G8 (anti-FcγRIIb) prior to addition of soluble immune complexes. Taken together, our experiments indicate that in unprimed neutrophils, soluble immune complexes bind to the cell surface via binding to FcγRII and this results in an increase in intracellular Ca²⁺; this interaction fails to activate the NADPH oxidase. Upon priming, the soluble complexes now generate an extra intracellular Ca²⁺ signal that arises via the functional activation of FcγRIIb. This extra Ca²⁺ signal arises from Ca²⁺ influx and requires protein-tyrosine phosphorylation. Experiments cross-linking individual receptors confirm these conclusions that ligation of FcγRII can elevate intracellular Ca²⁺ via internal store mobilization in either primed or unprimed cells, whereas ligation of FcγRIIIb can only elevate intracellular Ca²⁺ via Ca²⁺ influx in primed cells. If the traces of the intracellular Ca²⁺ transients generated via ligation of FcγRII and FcγRIIIb are combined, they closely resemble the kinetics of intracellular Ca²⁺ transients observed when primed and unprimed neutrophils are stimulated with soluble immune complexes. It was noteworthy that ligation of either FcγRII or FcγRIIIb failed to activate the synthesis of substantial levels of IP₃, in agreement with previous reports (31, 32).

There are numerous reports in the literature regarding the interaction of immune complexes with neutrophil Fc receptors, but none have addressed the interaction of soluble complexes with primed and unprimed cells. In our experiments, the intracellular Ca²⁺ transients generated by FcγRII ligation were partly sensitive to inhibition with tyrosine kinase inhibitors, confirming the recently reported association of FcγRII with tyrosine kinase activity (23, 24, 26, 27). Our data also confirm the importance of tyrosine kinase activity and FcγRIIIb function (27), and we propose that tyrosine kinase activity becomes associated with FcγRIIIb during priming and regulates Ca²⁺ influx. We speculate that during priming a Src-like tyrosine kinase (perhaps Hck; Ref. 27) becomes associated with FcγRIIIb, and this results in the assembly of a new signaling cassette linking FcγRIIIb ligation to cell activation. Several reports have shown that FcγRIIIb can transduce signals, including intracellular Ca²⁺ transients independently of FcγRII (28–30). A synergistic response of FcγRIIIb and CR3 has previously been reported, whereby CR3 immobilizes FcγRII to the plasma membrane via a cytoskeletal-dependent mechanism and then ligation of FcγRIIIb induces tyrosine activation in the proximity of FcγRII (25). In our experiments using synthetic soluble immune complexes, anti-CR3 antibodies do not affect the ability of primed neutrophils to activate a respiratory burst. Thus, the dependence of the response on CR3...
Neutrophil Activation by Soluble Immune Complexes

REFERENCES
