Calcium Changes in Immune Complex-stimulated Human Neutrophils
SIMULTANEOUS MEASUREMENT OF RECEPTOR OCCUPANCY AND ACTIVATION REVEALS FULL POPULATION STIMULUS BINDING BUT SUBPOPULATION ACTIVATION*

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Immune complexes (ICs) induce an initial transient increase in cytosolic intracellular calcium ([Ca$^{2+}$]$_i$) levels in human neutrophils (PMN). Changes in PMN [Ca$^{2+}$]$_i$ were measured with the fluorescent calcium indicator Indo-1 (1-[2-amino-5-(6-carboxyindol-2-yl)-phenoxy]-2-(2'-amino-5'-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid), at the level of individual cells by flow cytometry. Two kinds of immune complexes (ICs) were used in this study: an insoluble (IIC) and a more soluble less valent immune complex (SIC) with fewer available Fc receptor binding ends per molecule of SIC than IIC. Simultaneous binding and activation studies performed on the flow cytometer with fluoresceinated IIC or SIC demonstrated that a majority of the cells bound each stimulus uniformly. However, only an IC dose-dependent proportion of those IC-bound cells responded with an increase in [Ca$^{2+}$]$_i$. Analysis of Indo-1 fluorescence signals from neutrophils exposed to IIC, corrected for the contribution of the nonresponding population, indicated that every dose of IIC elicited a similar maximum [Ca$^{2+}$]$_i$ within the responding population. In contrast, the magnitude of the increase in [Ca$^{2+}$]$_i$ elicited by low doses of SIC did become dependent on dose. Cells treated with pertussis toxin and exposed to IIC exhibited a normal [Ca$^{2+}$]$_i$ response both in magnitude and expression. Therefore, [Ca$^{2+}$]$_i$ responses induced by immune complexes are expressed by subpopulations of PMN, in a response which is dependent on the valency of the stimulus. In addition, pertussis toxin sensitive G protein(s) appear not to have a major role in IIC-induced [Ca$^{2+}$]$_i$ changes, membrane potential changes, production of superoxide anions, and elastase release.

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human neutrophils (PMN) to exhibit increases in [Ca$^{2+}$]$_i$ (1, 2), which peak before the release of superoxide anion or granule contents (3, 4). Thus, transient increases in intracellular calcium ([Ca$^{2+}$]$_i$) have been implicated in the signal transduction induced upon PMN activation.

Three distinct subclasses of Fcγ receptors (FcγRI, FcγRII, and FcγRIII) have been identified on overlapping subsets of cells in the hematopoetic system, as well as on neutrophils (5). Freshly isolated PMNs express FcγRI and FcγRIII, whereas FcγRI appears only after long incubation with interferon γ (6). FcγRII and FcγRIII are structurally distinct, exist in multiple isoforms, and are reported to bind only multivalent immune complexes (5). The exact function of each Fcγ subclass in initiating and transducing signals in the PMN remains controversial (6–14). For example, some studies have implied that FcγRIII alone is important for phagocytosis (7), whereas others (8, 12, 14) find occupancy of both receptor subclasses is necessary to achieve this function. Ligation of PMN FcγRIII with an anti-FcγRIII monoclonal antibody, followed by cross-linking with a goat F(ab')$_2$ antimouse IgG has been reported to elicit a rise in cytoplasmic [Ca$^{2+}$] without occupancy of FcγRI (8), although both subclasses are said to be necessary to achieve an oxidative burst (5, 9, 10). Therefore, there is no agreement as yet on the role of each Fcγ subclass in immune complex-stimulated PMN responses.

There is also no agreement on the importance of receptor occupancy and stimulus valency (defined as the number of IgG molecules per immune complex) in the regulation of immune complex activation of PMN cellular responses (15). The crosslinking of cell receptors in other cells of the immune system is viewed as an important step in eliciting Fc receptor (FcR)-mediated responses (16–18), and it has been suggested that, similarly, cross-linking of FcR may explain the dependence of neutrophil stimulation on the IC valency (19–21).

The technique used in the present study uniquely approaches both of these questions, since it permits simultaneous correlation, for each cell, between receptor occupancy and the ensuing response.

It has been demonstrated that most if not all of the increase in phagocyte [Ca$^{2+}$]$_i$, caused by N-formyl peptides is mediated through pertussis toxin (PT)-inhibitable G proteins (22, 23). In contrast, Fc receptor-mediated responses may be activated through different signal transduction pathways depending on the valency of the stimulus. Thus, aggregated IgG-elicted

1 The abbreviations used are: PMN, human neutrophils; FcR, Fc receptor; PT, pertussis toxin; IC, immune complex; IIC, an insoluble IC; SIC, a more soluble less valent IC; F-IIC and F-SIC, fluoresceinated IIC and SIC, respectively; MCF, mean channel fluorescence; EGTA, [ethylenebis(oxyethylenenitrilo)tetraacetic acid; fMLP, formylmethionylleucylphenylalanine; FACS, fluorescence-activated cell sorter.

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responses have been reported to be sensitive to pertussis toxin treatment, whereas responses induced by IgG attached to a nonestigible solid surface are insensitive to this toxin (24, 25). These results, as well as others, suggest that, like platelets and muscle cells, both PT-sensitive and -insensitive receptor-ligand-activated signal transduction pathways may exist in PMN (26–28).

Flow cytometric analyses of changes in [Ca$^{2+}$]$_i$, have made use of the fluorescent Ca$^{2+}$ probe Indo-1 (3, 29). Indo-1 exhibits a shift in wavelength upon binding of Ca$^{2+}$ that permits the quantitation of [Ca$^{2+}$]$_i$ by using the ratio of the Ca$^{2+}$-bound and Ca$^{2+}$-free fluorescence emission peaks (30). Thus, the Indo-1 ratio is a measure of [Ca$^{2+}$]$_i$ that is independent of cell size or probe concentration. In addition, Indo-1 has the experimental advantages over Quin-2 of a high fluorescence yield and high K$_d$ that does not permit saturation of the dye at Ca$^{2+}$ concentrations achieved by activated cells (3, 31, 32).

The studies presented here document a dose-dependent disproportionation in the expression of the IC-induced PMN [Ca$^{2+}$]$_i$ response and investigate the roles of established mechanisms of IC activation in this response. To date, the use of simultaneous ligand labeling and cellular response in live cells has been limited. We make use here of simultaneous measurement of receptor occupancy and changes in [Ca$^{2+}$]$_i$ in individual cells, as observed by flow cytometry. Our results suggest that receptor occupancy is not sufficient for IC-induced activation of a cytoplasmic Ca$^{2+}$ transient and that such activation, once initiated, is independent of the dose, whereas the proportion of responding cells is dependent on dose. Stimulus of low valency initiated a smaller response than one of high Fc content/molecule of IC, but the expression of that response was still disproportionate. Finally our results indicate that this IC-induced [Ca$^{2+}$]$_i$ response is not dependent on pertussis toxin sensitive G proteins.

**MATERIALS AND METHODS**

**Neutrophil Isolation**—The isolation of neutrophils, from whole human blood collected in sodium citrate (0.38%), was performed as described initially by Boyum (33). Blood cells were separated from the platelet-rich plasma by centrifugation at 200 x g for 10 min at 25 °C. Red cells were sedimented by dextran at 1 x g for 30 min at 4 °C and the supernatant centrifuged at 200 x g for 10 min at 4 °C. The cell pellet was diluted with 0.154 mM NaCl and layered onto Ficoll Hypaque (Pharmacia, Sweden). The contaminating erythrocytes in the pellet were hypotonically lysed with water followed by 0.9% NaCl. This suspension was centrifuged at 200 x g for 10 min at 4 °C and the pellet brought up in phosphate-buffered saline (PBS) (125 mM NaCl, 2 mM Na$_2$HPO$_4$, 8 mM NaH$_2$PO$_4$, 5 mM KCl, and 5 mM glucose).

**Immune Complex Preparation**—Insoluble immune complex (ICC) was prepared with bovine serum albumin and rabbit anti-bovine serum albumin in a weight to weight ratio of 1:10 (molar ratio 1:4). This ratio represented the point of maximum precipitation (equivalence point) as measured by the precipitin curve (34). The appropriate amount of HSA (in H$_2$O) was added to anti-BSA (in 0.02 M PBS, pH 7.0) (Cappel Organon Technica, Durham, NC) and the solution incubated for 2 h at 37 °C and refrigerated overnight. The precipitate was washed three times with a 4-volume excess of PBS without glucose at pH 7.0, brought up in the same buffer, and used at a BSA-antibody protein concentration of 6 mg/ml as determined with the BCA protein assay kit (35).

A 1 mM mol ratio of BSA (2:1) was used to yield a more “soluble” immune complex preparation that was clear after centrifugation at 80,000 x g for 40 min. More soluble complexes were formed with BSA and rabbit anti-bovine serum albumin IgG fraction in a 1:2 antibody:antigen molar ratio and incubated at 37 °C for 2 h (Cappel Organon Technica, Durham, NC). This molar ratio fell within the zone of the excess of the precipitin curve, and therefore resulted in a soluble immune complex. A solution of IgG fraction prepared in parallel did not stimulate the PMNs. Concentrations of SIC used in the experiments were based on the concentration of anti-BSA, in order to be comparable with the concentrations of ICC.

**Preparation of Fluoresceinated ICC (F-ICC) and Fluoresceinated SIC (F-SIC)**—These ICC were prepared with fluoresceinated BSA. BSA was labeled with it with fluorescein isothiocyanate (Sigma) according to the method of Boyum et al. (36). The mixture was dialedyzed in a 100-fold larger volume of PBS without glucose, pH 7.0, for 3 days at 4 °C. The protein concentration was determined by the BCA method, with typical yields of ≥94% of the starting concentration of BSA.

**Loading PMN with Indo-1 Acetoxymethyl Ester**—Cells were loaded with 5 μM Indo-1 AM (from a 1 mM stock solution in dimethyl sulfoxide)/10 million cells/ml. Indo-1 AM freely passes into the cell and is hydrolyzed into its Ca$^{2+}$-sensitive state by the action of cytosolic esterases. The cells were incubated at 37 °C for 7 min and washed with a 5-fold excess of cold PBS and then centrifuged at 200 x g, at 4 °C, for 10 min (3).

**Measurement of [Ca$^{2+}$]$_i$, Simultaneously with Receptor Occupancy in Individual Cells by Flow Cytometry**—Flow cytometric analysis of individual cells was performed on a FACS 440 (Becton Dickinson, San Jose, CA) as described previously (3); the instrument is equipped with a dual laser and four photomultipliers, able to measure six parameters simultaneously. Data were collected and processed on a Microvax computer (Digital, Marlboro, MA). The secondary 4-watt argon laser was tuned to 557 nm to excite Indo-1, and emissions were collected simultaneously on linear scales, at 405 and 485 nm with 20- and 22-nm band pass filters, respectively. The appearance of bound F-ICC or F-SIC on the surface of cells was monitored by exciting the fluorescein group with a primary 2-watt argon laser tuned to 488 nm. Fluorescein emissions were collected at 530 nm with a 30-nm band pass filter. All FACS analyses of cells were performed on suspensions of 2 million cells/ml of Krebs-Ringer phosphate (KRP) (PBS + 1.5 mM MgSO$_4$. 0.9 mM CaCl$_2$), stirred, and maintained at 37 °C. The base-line fluorescence of unstimulated cells was collected before each cell stimulation. A sheath buffer of PBS without glucose at pH 7.4 was used.

**Calculation of [Ca$^{2+}$]$_i$, from Indo-1 Fluorescences**—Cell fluorescences were collected, each for 4000 cells, at 4-s intervals. The mean channel fluorescence (MCF) was determined by a computer from histograms depicting number of cells versus fluorescence channel. One can manually calculate a ratio $R'$ for each time point from the MCF at 485 and 405 nm, each representing an average fluorescence for 4000 cells (3). Recently, a ratio board has permitted the FACS computer to calculate the Indo-1 ratio for each cell individually by a mathematical process; the histogram for each 4000 cells time point then indicates the ratio $R'$ for each cell, each cell's MCF being converted to a mean ratio (MCF) rather than the ratio of the means. The scales of the two ratios, $R$ and MCF $R'$, are of necessity, different; they were therefore normalized, as indicated below. The Indo-1 ratio, calculated either way, was used in the Gryniewicz relationship: [Ca$^{2+}$]$_{i}$ = $K_d$ [Ca$^{2+}$]/($R' + R_d$) (30). The limiting ratios, $R_d$ and $R'$, were determined in digitonin-lysed cell suspensions using a 1 mM CaCl$_2$ solution or 10 mM EGTA solution in KRP, respectively. The constant $S$ is equal to the fluorescence of the Ca$^{2+}$-free dye at 485 nm, determined in a 10 mM EGTA solution of digitonin-lysed cells, divided by the intensity of the Ca$^{2+}$-saturated dye at 485 nm, determined in digitonin-lysed cell suspensions containing 1 mM CaCl$_2$. The published $K_d$ for Indo-1, 250 nM at physiological pH, was used for these calculations.

The $R'$, calculated from flow cytometric data, was similar to the ratios obtained in cell suspensions by spectrofluorimetry. The computer calculated ratio ($R'$) was presented as a histogram with a scale from 0 to 255 channels. In order to correlate the $R'$ to the MCF, the maximal Indo-1 ratio elicited by 10$^{-7}$ M fMLP was used as a reference point. This fMLP-induced Indo-1 ratio was chosen because it is equivalent to manually calculated Indo-1 ratios since ≥95% of the cells exhibit a [Ca$^{2+}$]$_i$ increase. The maximal fMLP-induced Indo-1 ratio exhibited by cells exposed to 10$^{-7}$ M fMLP was 190 ± 7, which is numerically 73 ± 5 times as large as the manually calculated $R'$ of 2.59 ± 0.04. The factor 73 was therefore used to normalize the $R'$ to the MCF to allow calculation of [Ca$^{2+}$]$_i$.

**Superoxide Anion Measurement**—Superoxide generation was measured by the lipoxygenase-inhibitable reduction of ferriochrome C type VI (Sigma) as described previously (37-39).

**Transmembrane Potential Change Measurement**—The lipophilic
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Changes in Cytosolic Calcium Induced by Insoluble Immune Complexes—Intracellular calcium changes in Indo-1-loaded cells were monitored on a flow cytometer, as described under "Materials and Methods," monitoring 4000 cells/4-s time point. Fig. 1A depicts a representative time course of the change in [Ca"²⁺\text{]_o}], as shown by changes in the calculated Indo-1 ratio, R. Every dose induced an initial increase in [Ca"²⁺\text{]_o}], from a base line of approximately 100 nM that reached a maximum and then fell to an above base-line value, equilibrating there within or after the 2-min time span of the experiment. The high dose, 240 μg/ml, of IIC caused a maximal calcium transient within 36 s. Progressively longer time intervals were required to reach maximum [Ca"²⁺\text{]_o}] with decreasing doses of IIC. The three highest doses of IIC (240, 120, and 60 μg/ml) induced similar maximal [Ca"²⁺\text{]_o}] of approximately 650 nM. The three lower doses of IIC (30, 15, and 5 μg/ml) caused maxima that were dependent on dose (approximately 380, 200, and 180 nM, respectively).

The unshaded individual Indo-1 ratio histogram in Fig. 2A shows that the mean computer calculated ratio, MCF, of the resting cell population was homogeneous. Upon stimulation with a saturating dose of 10^"⁻¹²" m fMLP, ≥95% of the population exhibited a maximal increase in [Ca"²⁺\text{]_o}], within 10 s. This response is depicted by the shaded peak in Fig. 2A. The fMLP-induced [Ca"²⁺\text{]_o}] response was consistently exhibited by ≥95% of the cell population, with similar maximal [Ca"²⁺\text{]_o}] values. Therefore, we used fMLP as a control for maximal cell activation in each experiment.

In contrast to the [Ca"²⁺\text{]_o}] response elicited by fMLP, IIC caused a partial population of PMN to exhibit maximal [Ca"²⁺\text{]_o}], whereas the remainder failed to respond (Fig. 2, B–D). The cells in the nonresponsive population were not dead, since 95% of them were shown to exhibit a maximal increase in [Ca"²⁺\text{]_o}] induced by the fMLP control. The proportion of cells exhibiting this increase in [Ca"²⁺\text{]_o}] was dose-dependent.

The correction of the overall Indo-1 signal for the contri-
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Fig. 2. Superimposed flow cytometric histograms of the individual cell Indo-1 ratio before stimulation and at maximum [Ca\(^{2+}\)]\(_{\text{i}}\). Histograms represent the number of cells (y axis) versus the individual cell Indo-1 ratios (x axis) for baseline (□) and for maximal stimulus-induced [Ca\(^{2+}\)]\(_{\text{i}}\) (■), using 10\(^{-7}\) M fMLP (A), 15 \(\mu\)g/ml IIC (B), 60 \(\mu\)g/ml IIC (C), and 240 \(\mu\)g/ml IIC (D). Data are representative of 10 separate experiments.

Fig. 3. The maximal [Ca\(^{2+}\)]\(_{\text{i}}\) increase in [Ca\(^{2+}\)]\(_{\text{i}}\)-responding cells exposed to various doses of SIC AND IIC. Data were calculated using maximal increases of [Ca\(^{2+}\)]\(_{\text{i}}\) elicited by ≥750 \(\mu\)g/ml SIC or 120 \(\mu\)g/ml IIC as 100% response and were from >3 separate experiments. * p < 0.05.

Simultaneous Flow Cytometric Measurement of Receptor Occupancy and Intracellular Calcium Changes—The existence of Ca\(^{2+}\)-responding and nonresponding subpopulations in the IIC-induced [Ca\(^{2+}\)]\(_{\text{i}}\) response could be due to cooperativity of IIC binding to the neutrophil. To investigate the role of receptor occupancy in initiating a [Ca\(^{2+}\)]\(_{\text{i}}\) change, the binding of ligand to PMN was monitored with a F-IIC. Changes in the Indo-1 ratio and fluorescein fluorescence elicited by F-IIC were measured simultaneously by flow cytometry (Fig. 4). The fluorescein fluorescence caused by ≥60 \(\mu\)g of F-IIC, attained its maximal value by the time maximal [Ca\(^{2+}\)]\(_{\text{i}}\) was reached (i.e. 30 s) (Fig. 4, A and B). The F-IIC-labeled cells exhibited

![Diagram](image-url)
a comparable percentage of Ca²⁺-responding and nonresponding cells and [Ca²⁺]i response to those induced by unlabeled IIC (Fig. 4, A and C). Doses of ≥60 µg of F-IIC-induced maximal binding of F-IIC and [Ca²⁺]i responses by 30 s. Lower doses of F-IIC, <60 µg/ml, displayed saturation of F-IIC binding and [Ca²⁺]i response more slowly but always by 50 s (Fig. 5).

At every dose of F-IIC, more cells bound F-IIC than responded to it with a [Ca²⁺]i transient (Fig. 6). The highest dose of F-IIC, 240 µg/ml, caused the maximum number of cells, 80%, to bind stimulus. Thus, as shown in Fig. 4, a [Ca²⁺]i responding and nonresponding population exists within a population of equally F-IIC-bound cells. Therefore, the existence of a disproportionate [Ca²⁺]i response cannot be explained by cooperativity of IIC binding to its receptor on responding cells, since equal binding to nonresponding and responding cells, alike, occurs.

**Activation of PMNs by Soluble and Particulate Immune Complexes** — The cross-linking of Fc receptors by ligand may be important in immune complex activation of PMN. The role of cross-linking immune complexes in eliciting [Ca²⁺]i responses was investigated by using an immune complex that was soluble and had a lower valency than IIC (see "Materials and Methods"). This IC theoretically should have a high number of single antibody molecules liganded to BSA bound on both antigen binding sites; therefore, this SIC should be less able than IIC to cross-link receptors.

Flow cytometric studies showed that SIC also elicited [Ca²⁺]i responses in a subpopulation of cells (Fig. 4D). Data corrected for the contribution of nonresponding cells showed that all doses of SIC elicited a [Ca²⁺]i that was maximal by 30 s (Fig. 1C). Furthermore, the magnitude of the response to [SIC] ≥480 mg/ml was similar to those elicited in responding cells by IIC. However, in contrast to IIC, <480 mg/ml SIC elicited maximal [Ca²⁺]i changes that were dependent on SIC dose (Fig. 3). We therefore investigated the role of receptor occupancy in SIC-induced [Ca²⁺]i responses, using a fluorescent SIC. These flow cytometric studies showed that the F-SIC bound maximally to ≥95% of the cells within the first 15 s of exposure (Fig. 4C). F-SIC bound uniformly to all the neutrophils in dose-dependent amounts but elicited a response in only a dose-dependent fraction of them (Fig. 4, C and D, and Fig. 7).

**The Effect of Blocking Pertussis Toxin-sensitive G Proteins on the IC-induced [Ca²⁺]i Response** — A known signal transduction pathway used by fMLP to elicit changes in [Ca²⁺]i involves pertussis toxin-sensitive G protein(s). These proteins have been implicated in the coupling of chemoattractant receptors to a phospholipase C, believed to be one of the first events in the chemoattractant signal transduction pathway (42). Therefore, the role of these G proteins in the signal transduction of the IC-induced [Ca²⁺]i transient was investigated.

The concentration of PT needed for complete G protein inactivation was investigated, using 0–1500 ng/ml PT, the range utilized by other investigators (23, 43–45). Up to 250 ng of PT/ml of cells was used in previous studies if the PT was pre-activated before addition to cells, whereas larger concentrations were needed if the toxin was to be activated by cellular processes. Our experiments allowed PT to be activated intracellularly by a 2-h incubation performed at 37 °C.

The amount of G protein modification by PT was indirectly measured by the inhibition of the fMLP-induced release of superoxide. Full abrogation of the release of superoxide in fMLP-stimulated cells was reached at 500 ng/ml PT. In

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**Fig. 5.** Time course representing the dose-dependent proportion of cells to which F-IIC bound as determined by flow cytometry. Indo-1-loaded cells were stimulated with 15–120 µg/ml F-IIC. The percent of the population binding stimulus was plotted versus time after stimulus addition. Cells binding F-IIC are defined as those displaying a fluorescent signal above channel 100 on the log scale. Arrows indicate the time of maximum [Ca²⁺]i, after addition of 120 (△), 60 (○), 30 (×), or 15 (■) µg/ml F-IIC. Data are representative of five similar FACS experiments.

**Fig. 6.** Expression of increases in [Ca²⁺]i within the F-IIC-labeled PMN population. ■, the percent of the whole population of cells that bound F-IIC; ○, the percent of the whole population that exhibited an increase in [Ca²⁺]i. All data represent mean ± S.E. and are from ≥3 separate experiments.

**Fig. 7.** Time course of F-SIC binding to PMNs at 37 °C measured by flow cytometry. Neutrophils were equilibrated in KRP at 37 °C for 3–5 min, a base-line autofluorescence emission taken, and then stimulus added. Bound fluorescence was determined by flow cytometry as described under "Materials and Methods." By definition, the autofluorescence of cells collected on the log scale has a linear value of 1 which is shown at time 0. Data represent the time course of binding 1260 (●), 960 (○), 480 (×), 60 (■), or 15 (△) µg/ml F-SIC. Data are representative of three separate experiments.
contrast, the superoxide release induced by IIC was 70% of that exhibited by buffer-treated (sham) cells. Higher concentrations of PT failed to inhibit further the release of superoxide elicited by IIC. Therefore, 500 ng of PT/ml of cells was used in all further experiments.

As others have also reported previously (25), we found a small proportion of cells (20%) which still retained a response to fMLP after PT treatment (compare Fig. 8, B and C). In contrast, virtually all of the PT-treated cells (>80%) retained the ability to respond to 240 µg/ml IIC equally as well as the sham cells (compare Fig. 8, E and F).

Taken together, these results indicate that the pertussis toxin-sensitive G proteins appear to have a minimal role in IIC-induced responses. To further test this hypothesis, IIC-induced membrane potential changes and release of elastase were investigated. Pertussis toxin treatment inhibited these by 22 ± 8% and 27 ± 5%, respectively.

**DISCUSSION**

As shown here, multiparameter flow cytometry kinetic analysis has permitted us to examine, simultaneously, agonist binding to and resultant response by human neutrophils. Whether the agonist was a particulate immune complex or a soluble complex, the binding to neutrophils was uniform (Fig. 4, A and D). In spite of this uniform binding (which can be considered as receptor occupancy), only a subpopulation of the complex-ligated cells responded with a [Ca2+]in transient (Fig. 6), an event which is considered part of the intracellular signal transduction mechanism (1-3, 46, 47). The proportion of cells so responding depended upon the dose (Fig. 6), but, interestingly, the magnitude of the [Ca2+]in transient response was independent of IIC dose but dependent on the SIC dose (Fig. 3). The maximal [Ca2+]in transient elicited by 60 mg/ml of agonist was three times higher for the particulate IIC than for the soluble SIC (Fig. 1, B and C), indicating the importance of Fc receptor cross-linking in eliciting cytoplasmic [Ca2+]in. This mechanism appears to be stimulus and receptor-dependent since cells exposed to the soluble chemoattractant, fMLP, exhibit a maximal [Ca2+]in in >95% of the population regardless of dose (Fig. 2A) (3).

One possible explanation for the appearance of responding and nonresponding [Ca2+]in populations in cells exposed to IIC could have been that the stimulus preferentially binds to some of the cells due to cooperation between Fc receptors. Our studies were designed to address the possible roles of Fc receptor cooperativity by simultaneous analysis of F-IIC binding and the ensuing changes in [Ca2+]in. These studies demonstrated that a significant population (>80%) bound F-IIC uniformly (i.e. to the same extent) within 20 s after exposure (Figs. 4 and 5). The extent of receptor occupancy, as indicated by the uniform stimulus binding, was dose-dependent and identical for both the responding and nonresponding populations (Fig. 4). However, only a proportion of the cells binding the F-IIC responded with an increase in [Ca2+]in (Fig. 4). As noted above, the responding population [Ca2+]in increase was similar at every dose of IIC used. These results suggest that receptor binding cooperativity does not have a role in determining which neutrophil will respond to IIC with a [Ca2+]in transient, nor does the IIC dose determine the magnitude of that response which appears, rather, to be an "all or none" response.

Studies comparing the [Ca2+]in response elicited by insoluble IC, capable of cross-linking receptors, versus soluble IC, less capable of cross-linking receptors, showed that the magnitude of this response was dependent on the size and valency of the complex. Flow cytometric studies showed that the magnitude of the responding cells' maximum [Ca2+]in, elicited by >480 µg/ml SIC, was similar to that elicited by any dose of IIC (5-240 µg/ml) (Fig. 2C). However, cells responding to concentrations of SIC below a saturating dose exhibited a smaller SIC dose-dependent change in [Ca2+]in (Fig. 2C) which may indicate a different mechanism of Ca2+ signal propagation from that exhibited by IIC. There is precedence for the conclusion that different stimulus valencies may activate cells by different pathways (65).

Soluble immune complex (15-1260 µg/ml), as well as insoluble immune complex, stimulated a rise in [Ca2+]in, in a subpopulation of the cells, although all bound the ligand uniformly. Therefore, the appearance of such a disproportionation in the PMN response is not dependent on the cross-linking of the FcR receptors.

Cross-linking of Fc receptors by ligands has been recognized as one mechanism for initiation of cellular responses (10, 20, 47-50). Therefore, our studies are in accord with other myeloid cell studies that correlate increased aggregation of Fc receptors by immune complex with increasing valencies inducing higher [Ca2+]in responses (11, 21). The data presented in Figs. 3A and 7 indicate that only the lowest doses of SIC (15 and 60 µg/ml) induce a maximum [Ca2+]in that is dependent on the amount of SIC bound. These results imply the
existence of a \([Ca^{2+}]_m\) response threshold, whose magnitude may be dependent on stimulus valency but not on Fc receptor occupancy by multivalent IIC.

The magnitude of the fluorescence from the F-SIC was used as a measure of the extent of SIC binding to PMN. Cells exposed to 960 \(\mu g/ml\) SIC exhibited saturation of SIC binding sites and maximal \([Ca^{2+}]_m\) transients, whereas half that concentration of F-SIC bound fewer sites but still elicited the same \([Ca^{2+}]_m\) (Figs. 3 and 7). These results indicate that the magnitude of the \(Ca^{2+}\) response, and the number of available stimulus binding sites may not be satisfied at equivalent stimulus concentrations. Therefore, the extent of the IIC-elicted change in \([Ca^{2+}]_m\), in the neutrophil is dependent on the valency of the stimulus, whereas the initiation of the \(Ca^{2+}\) response depends on a function other than receptor occupancy. This function is not related the ability of immune complexes to cross-link receptors, since both F-IIC and F-SIC liganded cells exhibited a responding and nonresponding \(Ca^{2+}\) populations (Fig. 4). Thus our studies of IIC and SIC demonstrate that, except for low doses of SIC, both SIC and IIC can elicit similar disproportionately expressed increases in \([Ca^{2+}]_m\) and that, although receptor occupancy is necessary for activation, such occupancy is not sufficient to achieve it.

Furthermore, the \([Ca^{2+}]_m\) transient, when it does occur, is maximal and is not stimulus dose-dependent, whereas the fraction of cells (the subpopulation) which responds to the stimulus is dependent on its dose. Although both IIC and SIC were able to elicit a \([Ca^{2+}]_m\) transient, SIC was unable to elicit changes in membrane potential, production of superoxide, and release of elastase, all of which result from IIC-induced activation.

Some have suggested that, based on cytochemical evidence, there exist subpopulations of neutrophils dependent on PMN maturity, Fc receptor subclass function and distribution, granule contents, distinct expression of receptors as identified by monoclonal antibodies, or previous exposure to cytokines (12, 51–57). Were this the explanation of our findings, we would expect large donor differences. These were not found; on the contrary, our findings appear to be reproducible for the same dose of IIC (or of SIC). It should be noted that functional PMN subpopulations based on oxidative burst but not on \(Ca^{2+}\) transients have been identified (58, 59) and that, clinically, shifts in oxidative burst and phagocytosis by PMN subpopulations appear to be important in bacteremia and hypergammaglobulinemia, respectively (58, 60).

Only with the advent of fluorescence imaging and of flow cytometry has it been possible to correlate a response with the actual receptor occupancy on the responding cell. It has been reported that large particulates, such as \(Candida albicans\) hyphae, can elicit \([Ca^{2+}]_m\), in subpopulations within a uniformly liganded PMN population (61). Human platelets stimulated with thrombin also exhibit \([Ca^{2+}]_m\) responses in subpopulations, even though thrombin is uniformly bound to all the cells (32, 62, 63). At submaximal physiological doses of thrombin, a maximal \([Ca^{2+}]_m\) response occurs in only a portion of the platelets, whereas the others remain at resting \([Ca^{2+}]_m\) levels (62). Therefore, functional subpopulations may have an important place not only in clinical disorders but also in the regulation of normal cell function.

Flow cytometric studies indicated that cells treated with pertussis toxin exhibited no inhibition of the IIC-induced \(Ca^{2+}\) response (Fig. 8). Studies performed at the level of the individual cell showed that the \(Ca^{2+}\) response elicited by IIC was disproportionate, as in untreated normal cells, implying no alteration in FcR function (Fig. 8, D and E). IIC induced similar \(Ca^{2+}\)-responding population proportions in both the PT-treated and untreated cells (Fig. 8, E–G). These results implied no alteration of Fc receptor affinity in PT-treated cells. Therefore, the magnitude and expression of the \([Ca^{2+}]_m\) response is independent of pertussis toxin-sensitive G proteins. Our results also demonstrate that PT treatment has only a small (<30%) inhibitory effect on the IIC-induced change in neutrophil: transmembrane potential, oxidative burst, and elastase release. Most of the IIC-induced activation may therefore either be transduced through a pertussis toxin-insensitive G protein, as suggested by the theory that signal transduction utilizes a family of G proteins, some of which are not sensitive to pertussis toxin (8, 59) or, alternatively, may use an entirely different mechanism of transduction, such as a calmodulin-regulated enzyme pathway (26, 27, 65).

In contrast to our results with immune complexes, Fiester et al. (9) indicate that superoxide production induced by the ligation of FcR\(_\text{IIa}\) is fully sensitive to pertussis toxin. One explanation for this discrepancy could be that immune complexes initiate both superoxide and degranulation through both FcR subclasses, and we have preliminary data, supporting this view, that both FcR subclasses are needed for immune complexes to induce a full oxidative burst (69).

Another explanation might be, as indicated by Crockett-Torabi and Fantone (13), that different conformations of FcR ligands may initiate activation by different pathways; in this context, ligation of FcR subclasses by anti-mouse Fab\(_\text{a}\) involves a ligand which differs in conformation from multivalent immune complexes and may not activate the signal transduction by the same mechanism we are studying here.

Our results therefore agree with those published by Blackburn et al. (64) and Kimberly et al. (7) which found FcR-mediated induction of an oxidative burst to be insensitive to pertussis toxin treatment but disagree with the report by Fiester et al. (9) that superoxide production initiated by binding to FcR\(_\text{IIa}\) is completely pertussis toxin inhibitable. One explanation for the latter discrepancy might be that superoxide production can be mediated through either receptor, as has been implied elsewhere (10, 13), whereas another possible explanation is the difference between the Fc ligands used in their and our studies, which could function by different activation mechanisms; they used a monoclonal antibody to the receptors, followed by cross-linking with anti-mouse IgG, whereas we use a multivalent immune complex and thus need no secondary antibodies; the exact level of receptor cross-linking achieved with these inherently different stimuli has not been evaluated.

Our flow cytometric studies of pertussis toxin-treated cells found that the fMLP-induced \(Ca^{2+}\) response was inhibited by 70%, and the proportion of cells exhibiting this response was halved (Fig. 5). These results support findings by others (23) that residual \(Ca^{2+}\) transients can be induced by fMLP in PT-treated cells. This appearance of a residual fMLP-induced \(Ca^{2+}\) response is thought to imply an alteration of receptor affinity by pertussis toxin treatment (46, 66). A similar alteration of affinity of Fc receptors by pertussis toxin treatment seems unlikely, since our data show no change in the binding of IIC, in the magnitude of response or in the number of cells exhibiting a \([Ca^{2+}]_m\) response to IIC (Fig. 8).

Other studies performed in this laboratory suggest that the coupling mechanism for particulate stimuli, such as opsonized zymosan and opsonized \(C. albicans\) hyphae, may partially depend on a pertussis toxin-inhibitable G protein. These stimuli were still able to elicit changes in \([Ca^{2+}]_m\), membrane potential, and to produce superoxide after toxin pretreatments (28). Particulate stimuli which induce cross-linking of receptors on the PMN, such as concanavalin A, have been reported...
to function normally on pertussis toxin-treated PMN, eliciting superoxide and myeloperoxidase release (67). These findings and the unclutered above support the conclusion that some, but not exclusively pertussis toxin-sensitive G protein-mediated transductional pathways may be initiated by Fc receptors. It has been reported that chemoattractants may also initiate neutrophil activation by more than one signal transduction pathway (45, 68).

These experiments agree with and extend findings of major dissimilarities between IIC and MLLP PMN activation pathways by demonstrating differences in the mechanisms of signal initiation, in the rate and magnitude of response, and in the expression of the response in the whole population. Therefore, the regulation of Fc receptor cell activation can occur at the level of receptor binding, aggregation, and unique signal transduction mechanisms. Simultaneous measurement of receptor occupancy and cell activation on the individual cell level has allowed us to probe the individual cell requirements for activation through the Fc receptor. The regulation of activation by the Fc receptors is under investigation.

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