The Journal of Immunology

RESEARCH ARTICLE | JANUARY 15 1991

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B A Walker; ... et. al

J Immunol (1991) 146 (2): 735–741.
https://doi.org/10.4049/jimmunol.146.2.735

0022-1767/91/1462-0735$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 146, 735–741, No. 2, January 15, 1991
Printed in U.S.A.

SIGNAL TRANSDUCTION EVENTS AND FcγR ENGAGEMENT IN HUMAN NEUTROPHILS STIMULATED WITH IMMUNE COMPLEXES[1]

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Signal transduction events have been evaluated in human neutrophils stimulated with immune complexes consisting of polyclonal rabbit antibody complexed with BSA. Immune complexes induced dose-related O_2^- responses, but very small increases in intracellular calcium ($[Ca^{2+}]_i$) levels were observed, in contrast to FMLP-stimulated cells. Measurements employing [$^{45}Ca^{2+}$] demonstrated that calcium influx and efflux in cells stimulated with immune complexes was substantially less than fluxes found in FMLP-stimulated cells. With respect to inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) formation under conditions in which the O_2^- responses to immune complexes or FMLP were similar, the $Ins(1,4,5)P_3$ response to immune complexes was much smaller (by 65%) as compared to that induced by FMLP. Although pertussis toxin-treated cells showed a greatly diminished O_2^- response (by 89%) to FMLP, the response to immune complexes was largely resistant (only 26% reduction) to the inhibitory effects of this toxin. Antibodies to FcγR indicated that engagement of FcγRII and FcγRIII, but not FcγRI, receptors was related to the O_2^- response of neutrophils to immune complexes. O_2^- formation occurred in neutrophils incubated with *Staphylococcus aureus* cell walls bearing antibodies to FcγRII or FcγRIII. These data indicate that, in human neutrophils stimulated with immune complexes, signal transduction events involve engagement of FcγRII and FcγRIII. The O_2^- response is largely pertussis-toxin insensitive, is not associated with a significant increase in levels of $[Ca^{2+}]_i$, and is associated with relatively little formation of $Ins(1,4,5)P_3$. This is in contrast to cells stimulated with FMLP in which O_2^- responses are largely pertussis toxin-sensitive and associated with large increases in $[Ca^{2+}]_i$ as well as formation of $Ins(1,4,5)P_3$. Signal transduction events involving FcγR appear to be quite different from those events related to engagement of FMLP receptors.

Phagocytic cells respond to soluble and insoluble stimuli with a respiratory burst that is associated with the production of oxygen-dependent products containing microbicidal activity (1–5). Signal transduction events related to this phenomenon have been studied fairly extensively in neutrophils stimulated by the chemotactic peptide FMLP. There is abundant evidence that FMLP interacts with neutrophils in a manner that leads to motility (chemotaxis) (6, 7), enzyme secretion (6, 8, 9), and production of O_2^- (6, 10). These receptor-mediated responses (11–13) require the engagement of a pertussis toxin-sensitive g-binding protein (reviewed in References 11 and 14), resulting in the production of $Ins(1,4,5)P_3$[3] (15–18) and DAG (19–21). Elevations in $Ins(1,4,5)P_3$ are associated with an increase in levels of $[Ca^{2+}]_i$ (22). DAG formation results in activation of protein kinase C and stimulation of NADPH oxidase (23–25).

It is known that immune complexes containing polyclonal rabbit IgG antibody to the Ag, BSA, represent a potent stimulator of O_2^- production by human neutrophils (26, 27) and that this process of cell activation requires an intact Fc region of the IgG molecule (27). This implies that immune complexes stimulate O_2^- production in neutrophils via signal transduction pathways that are dependent on receptor recognition of Fcγ. At least three separate FcγR are present on human neutrophils, FcγR, FcγRII, and FcγRIII, with the last being predominant in terms of receptor density (28). The purpose of this current study was to compare signal transduction events in human neutrophils stimulated with immune complexes to those induced by FMLP. The unexpected observations in these studies suggest that signal transduction events in neutrophils stimulated with immune complexes may be fundamentally different from signal transduction events in FMLP-stimulated neutrophils.

MATERIALS AND METHODS

Reagents. All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) except where noted. Cytochalasin B (2.5 μg/ml) was used only in selected experiments as described in the text. mAb 32.2, IV.3, and 3G8, as intact molecules or as the Fab or $F(ab')_2$ fragments, were purchased from Medarex (W. Lebanon, NH). Each of the fragments used showed a spectrum of receptor-binding activity similar to the parent compound, as determined by flow cytometry with the use of human neutrophils (or IFN-γ-treated neutrophils in the case of 32.2). At concentrations of 20 μg/ml, each antibody preparation (32.2, IV, and 3G8) caused >90% saturation of binding sites on human neutrophils, as assessed by immunofluorescence in flow cytometry. In the case of 32.2 antibody, neutrophils were pretreated with IFN-γ for full epitope expression.

Received for publication June 19, 1990.
Accepted for publication October 26, 1990.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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[3] Abbreviations used in this paper are: $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; $[Ca^{2+}]_i$, intracellular calcium; SOD, superoxide dismutase; g-binding protein, guanine nucleotide binding protein; DAG, diacylglycerol.

Agonists. The chemotactic peptide FMLP (10 mM) and PMA (2mg/ml) were stored frozen in DMSO and diluted as needed. Immune complexes were freshly made for each experiment by mixing BSA with rabbit polyclonal anti-BSA IgG antibody (Cappel Laboratories, West Chester, PA) in an Ag:antibody ratio of 1:5 (w/w) in order to obtain precipitating complexes at the point of Ag equivalence (29). Complexes were allowed to form (in the absence of serum) for 30 min before dilution and use. For a limited number of experiments, heat-aggregated human IgG was prepared for each experiment by heating a solution of IgG (18 mg/ml) (Cappel) at 62°C for 20 min. The preparation was allowed to cool to room temperature and diluted before use.

Preparation of neutrophils. Neutrophils were prepared as previously described (29). Briefly, whole citrated blood was centrifuged at 400 × *g* for 15 min and the platelet-rich plasma removed. The remaining pellet was diluted with PBS (140 mM NaCl, 1 mM KH_2PO_4, and 5 mM $NaPO_4$, pH 7.3), layered onto Ficoll-Hypaque, and centrifuged (400 × *g*, 30 min, 22°C). The lower layer was removed and the RBC were lysed twice with 150 mM NH_4Cl. The remaining neutrophils were washed and resuspended in ice-cold Tris-buffered HBSS and stored on ice until use.

Measurement of superoxide responses. Neutrophils (2×10^6) were stimulated by the addition of 100 μl of agonist in a total reaction volume of 1.0 ml HBSS containing 80 μM cytochrome *c* (horse heart, type III). For kinetic measurements, changes in OD were then measured eight times/min at 550 nm in a Gilford Response II spectrophotometer (Ciba-Corning Diagnostics Corp., Oberlin, OH). Changes in OD were calculated relative to the time of addition of agonist and to reaction mixtures containing SOD (85 U/ml). Production of O_2^- was calculated by using the extinction coefficient for cytochrome *c*, 18.5 cm^{-1} M^{-1}. For assays in which cells pretreated with pertussis toxin were employed, generation of O_2^- over a 30-min period was determined according to previously described procedures (30). Prewarmed (37°C) cells (2×10^6) were added to reaction mixtures (final volume of 1.0 ml) containing 80 μM ferricytochrome *c* ± SOD (85 U/ml) and cytochalasin B (2.5 μg/ml) where indicated. The samples were incubated for 4 min at 37°C followed by addition of agonist. After 30 min at 37°C the samples were diluted with 800 μl of HBSS and the cells removed by centrifugation. The OD was read immediately at 550 nm and amounts of O_2^- generated were calculated as described elsewhere (29).

To determine the relative role of each FcγR in the immune complex response, neutrophils (2.4×10^7/ml) were exposed to 4 μg/ml of the mAb 32.2 (anti-FcγRI(CD64), IgG1), IV.3 (anti-FcγRII(CD32), IgG2b), or 3G8 (anti-FcγRIII(CD16), IgG1) for 10 min at 23°C. Cells were then diluted to 2×10^6/ml, immune complexes were added, and the O_2^- response was measured. For some experiments, Fab (IV.3) or $F(ab')_2$ (32.2, IV.3) fragments of the mAb were used. In some experiments, cells pretreated with mAb were pelleted and resuspended in buffer containing 4 μg/ml of polyclonal goat IgG $F(ab')_2$ fragment specific for mouse IgG (Cappel) for 10 min before dilution and use as above.

For direct FcγR-specific stimulation of neutrophils, cells were exposed to formalin-fixed *Staphylococcus aureus* that had been coated with IV.3, 3G8, MPOC 21 (mouse monoclonal IgG1), or MOPC 141 (mouse monoclonal IgG2b). Coated particles were prepared by mixing (1 h, 23°C) 1 μg of antibody with 10 μl of a 10% (v/v) slurry of washed formalin-fixed *S. aureus* particles. This suspension was then diluted and used with final concentrations of 2 μg/ml antibody and 0.2% (v/v) *S. aureus* (9×10^7 particles/ml).

Pertussis toxin treatment. Neutrophils (2×10^7/ml) suspended in buffer (150 mM NaCl, 5.6 mM KCl, 500 μM $CaCl_2$, 200 μM $MgCl_2$, 1.0 mM glucose, 10 mM HEPES, 0.1% (w/v) BSA, and 50 U/ml DNase I) with or without pertussis toxin (2 μg/ml; List Biological Laboratories, Campbell, CA) were incubated with gentle agitation for 2 h similar to that described elsewhere (31). Neutrophils were then centrifuged and resuspended to give the same concentration of cells in each of the two groups. Treatment in this manner resulted in no appreciable loss of cell number and >90% of the neutrophils in each group excluded trypan blue dye.

Measurements of changes in $[Ca^{2+}]_i$. Measurements of $[Ca^{2+}]_i$ using the fluorescent probe fura-2 has been described previously (32). Briefly, neutrophils were suspended in buffer (150 mM NaCl, 5.6 mM KCl, 2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.0 mM glucose, and 10 mM HEPES, pH 7.3), loaded for 15 min at 37°C with 2 μM fura-2/AM (the acetoxymethyl ester of fura-2; Calbiochem, La Jolla, CA), and then for an additional 15 min after a 10-fold dilution. The cells were then filtered through gauze to remove cell aggregates, centrifuged (400 × *g*, 6 min) at 5°C, resuspended in buffer, and stored at 23°C until use. Similar studies were performed with quin-2 (Calbiochem). Neutrophils were resuspended in buffer at 10^8 cells/ml, incubated for an additional 20 min at 37°C with 50 μM quin-2/AM followed by a 10-fold dilution and incubation for 20 min. The cells were then centrifuged (400 × *g*, 6 min at 5°C), resuspended at 10^7 cells/ml in buffer, and maintained at 23°C until use.

Changes in $[Ca^{2+}]_i$-dependent fluorescence were measured in a Perkin-Elmer (Norwalk, CT) LS-5B luminescence spectrometer by using a thermally equilibrated cuvette holder at 37°C, with excitation/emission wavelengths of 342/500 nm for fura-2 and 339/492 nm for quin-2. Levels of $[Ca^{2+}]_i$ were determined by using the equation:

$$[Ca^{2+}]_i = \frac{K_d \times (F - F_{min})}{(F_{max} - F)}$$

Where F_{max} represents the maximum fluorescence after the lysis of cells with 0.2% Triton X-100, F_{min} represents the fluorescence after the addition of EGTA (10 mM, pH 10.5) and the K_d is 224 nM in the case of fura-2 and 120 nM in the case of quin-2.

$[^{45}Ca^{2+}]$ influx and efflux. Assays of $[^{45}Ca^{2+}]$ flux were performed in a manner similar to Korchak et al. (33, 34). For uptake assays, purified neutrophils were equilibrated in buffer (150 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 400 μM $CaCl_2$, and 10 mM HEPES, pH 7.45) at 37°C for 5 min. $[^{45}Ca^{2+}]$ uptake was initiated by the addition of prewarmed stimulus or buffer containing 150 μCi/ml $^{45}CaCl_2$ (Amersham Corporation, Arlington Heights, IL) and terminated after 5 min by the addition of 10 mM EGTA (pH 7.4). Aliquots of cell suspensions were immediately layered over silicon oil and centrifuged in a microfuge for 20 s and the radioactivity of the pellet was determined.

For efflux assays, neutrophil suspensions were incubated in uptake buffer containing 150 μCi/ml $^{45}CaCl_2$ for 45 min. Preliminary experiments indicated that isotopic equilibrium was achieved by 45 min. After the loading procedure, cells were washed three times in room temperature buffer, resuspended, and preincubated at 37°C for 5 min. Efflux was initiated by the addition of an aliquot of the cell suspension to the stimulus or buffer and terminated by centrifugation through silicon oil after which the radioactivity of the supernatant was determined. Results for both influx and efflux are expressed as the fold increase over basal (unstimulated) levels.

Ins(1,4,5)P_3 mass. Neutrophils (2×10^7/ml) were stimulated with agonist at 37°C and 100-μl aliquots were added to an equal volume of TCA (20% w/v) at 10-s intervals after addition of agonist. After 15 min at 4°C, the TCA-insoluble material was removed by centrifugation and the supernatant was washed five times with 1.0 ml of water-saturated diethyl ether and the aqueous layer was neutralized with 1 M $KHCO_3$. The final neutralized aqueous sample was dried under vacuum (Speed Vac, Savant Instruments, Farmingdale, NY) and stored at −20°C until assayed. Ins(1,4,5)P_3 mass was determined by using a stereospecific radioligand-binding assay (Amersham Corporation) (35). Based upon pilot experiments examining Ins(1,4,5)P_3, levels over 10 min after the addition of agonists peak Ins(1,4,5)P_3 levels were defined for each agonist as the average of the two highest Ins(1,4,5)P_3 levels at adjacent 10-s time points.

Statistics. The data were expressed as the mean ($\bar{x}$) and SEM. A paired *t*-test was used to compare the response between two treatments and statistical significance was defined at $p < 0.05$.

RESULTS

Superoxide responses. Agonists for generation of O_2^- in human neutrophils included 100 nM FMLP, insoluble immune complexes (consisting of 60 μg polyclonal rabbit IgG antibody to BSA and 12 μg BSA/ml), and heat-aggregated human IgG (100 μg/ml). Doses were selected such that the amounts of agonists employed would give a similar O_2^- response. In the mean of five experiments, immune complexes and FMLP caused generation of 12.6 ± 2.0 and 15.7 ± 3.1 nmol O_2^-/30 min, respectively, whereas heat-aggregated IgG was slightly less effective (9.7 ± 2.3 nmol O_2^-/30 min). As shown in Figure 1, the O_2^- response to FMLP reached a plateau within 2 to 3 min, whereas the response to immune complexes showed a slower initial rate, but in contrast to the effects of FMLP, production of O_2^- was largely sustained over the 30-min period. In the case of heat-aggregated IgG, there was a long lag period (5 to 8 min) followed by a sustained period (for at least 20 min) of O_2^- production.

$[Ca^{2+}]_i$ Changes. $[Ca^{2+}]_i$ changes were assessed in fura-2-loaded human neutrophils stimulated with the agonists described above. Figure 2 is representative of results from five separate experiments, showing the continuous

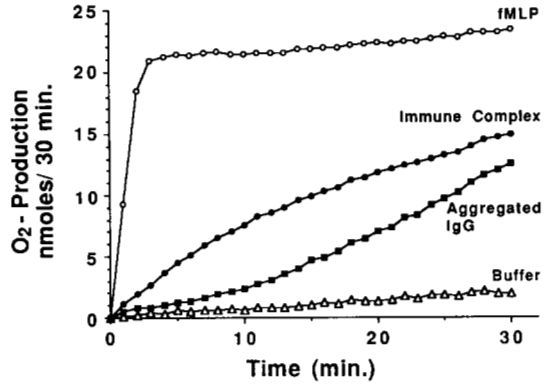

Figure 1. Time course (continuous spectrophotometric recording) for superoxide production in 2×10^6 neutrophils exposed to 100 nM FMLP, immune complexes containing 60 μg/ml antibody, or 100 μg/ml heat-aggregated human IgG. The vertical axis is the computed O_2^- generation defined by SOD-inhibitable reduction of ferricytochrome *c*. The recording represents an automatically calculated and plotted difference between cuvettes containing or lacking SOD.

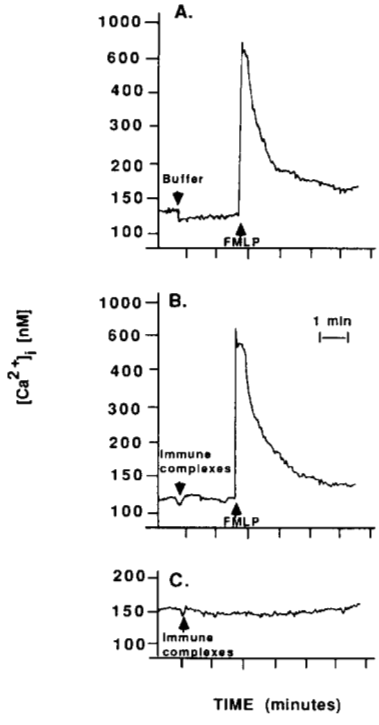

Figure 2. Continuous spectrofluorometric recording of fura-2-loaded neutrophils stimulated with immune complexes or FMLP. *A*, buffered salt solution was added followed 2.5 min later by FMLP (100 nM); *B*, immune complexes (60 μg antibody/ml) were added followed by FMLP (100 nM) at 2.5 min; *C*, only immune complexes (60 μg antibody/ml) were added and the $[Ca^{2+}]_i$-dependent fluorescence was followed for 6 min.

recording of $[Ca^{2+}]_i$-dependent fluorescence. As is evident in *A*, the increase in $[Ca^{2+}]_i$ after the addition of FMLP (100 nM) was immediate, reaching a peak within the first 20 s after addition of the peptide. In *B* and *C*, it can be seen that the exposure of neutrophils to immune complexes (60 μg antibody/ml) produced very small, if any, increases in $[Ca^{2+}]_i$, in spite of the fact that the subsequent addition of FMLP to the same cell preparation at 2 min (*B*) produced a calcium transient that was equivalent to the FMLP control (*A*). Similar responses of neutrophils loaded with quin-2 have also been found, ($n = 3$, data not shown). When increases in $[Ca^{2+}]_i$ in fura-2-loaded neutrophils were carefully evaluated in five separate experiments, exposure of neutrophils to immune complexes did not significantly increase $[Ca^{2+}]_i$ nor alter the response to FMLP (data not shown). Extending the time of recording to 20 min still failed to evoke an increase in $[Ca^{2+}]_i$ after addition of immune complexes (data not shown). Thus, the failure to detect changes in $[Ca^{2+}]_i$ in immune complex-stimulated cells is not caused by an artifact that precludes detection of changes in fluorescence. When similar studies were done with 100 μg/ml human heat-aggregated IgG or with BSA/anti-BSA immune complexes with Ag:antibody ratios ranging from 1:1 to 1:20 (containing 60 μg antibody/ml), no increase in $[Ca^{2+}]_i$ was noted. These same cells responded to addition of 100 nM FMLP with a full increase in $[Ca^{2+}]_i$ (data not shown).

The relationship between changes in $[Ca^{2+}]_i$ and O_2^- responses in cells stimulated with a range of concentrations of immune complexes or with FMLP is shown in Figure 3. The O_2^- response to immune complexes was dose dependent. At concentrations of immune complex exceeding 100 μg antibody/ml, no further increase in O_2^- occurred. At these concentrations, the O_2^- response appeared to be slightly attenuated. Over this dose range, very little increase in $[Ca^{2+}]_i$ was detected (Fig. 3*A*). In contrast, when FMLP was used as the agonist, there was an increase in the levels of $[Ca^{2+}]_i$ at concentrations of FMLP as low as 250 pM; a plateau of peak $[Ca^{2+}]_i$ was reached with 1 nM FMLP. The O_2^- response to FMLP was associated with intracellular levels of calcium reaching a plateau value of 719 nM calcium (Fig. 3*B*).

Flux measurements utilizing $[^{45}Ca^{2+}]$ were also carried out in immune complex- and FMLP-stimulated cells by using conditions similar to those described in Table I.

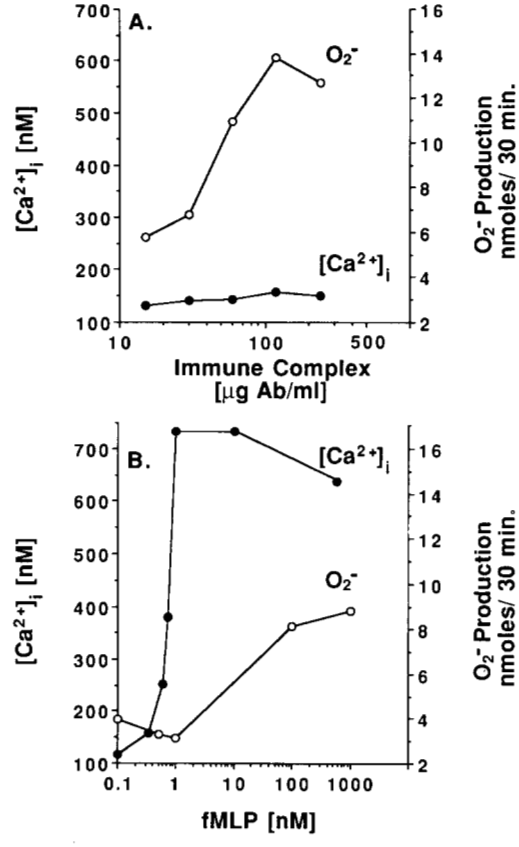

Figure 3. Dose-response relationships between concentrations of agonists (*A*, immune complexes; *B*, FMLP), O_2^- responses (nmol O_2^-/30 min/ 2×10^6 cells) and changes in $[Ca^{2+}]_i$.

TABLE I

[$^{45}Ca^{2+}$] flux across intact neutrophil cell membranes

Agonist	Dose	Influx[a] Fold increase ($\bar{x}$ ± SEM)		Efflux[a] Fold increase ($\bar{x}$ ± SEM)	
None		1.0		1.0	
FMLP	100 nM	5.24 ± 1.53	$p < 0.03$	5.39 ± 0.63	$p < 0.03$
Immune complexes	60 μg antibody/ml	2.31 ± 1.02		2.01 ± 0.19	

[a] Fold increase in [$^{45}Ca^{2+}$] flux as compared with unstimulated cells. Data are derived from four separate experiments. Actual mean counts per minute/10^6 neutrophils for basal (nonstimulated cells) [$^{45}Ca^{2+}$] influx in each of four separate experiments were 546, 5698, 2812, and 2561. For basal efflux values the mean counts in each of four separate experiments were 452, 543, 476, and 670 cpm/10^6 neutrophils.

Both influx and efflux of [$^{45}Ca^{2+}$] were measured according to established techniques (33, 34). Because the actual amount of radioactivity varied considerably from experiment to experiment, the data in Table I are represented as fold increase in radioactivity ($n = 4$). On the basis of [$^{45}Ca^{2+}$] influx measurements, it can be seen that 100 nM FMLP caused a 5.24-fold increase in the entry of calcium into neutrophils whereas immune complexes containing 60 μg antibody/ml showed a significantly lower (by 76%) [$^{45}Ca^{2+}$] influx ($p < 0.03$). With respect to measurements of [$^{45}Ca^{2+}$] efflux, the data also demonstrate a similar pattern; compared with the effects of FMLP, immune complexes caused much less calcium efflux from neutrophils. These data appear to be consistent with the differences in changes in levels of $[Ca^{2+}]_i$ with the use of the fura-2 or quin-2 probes in neutrophils stimulated with FMLP or immune complexes.

Ins(1,4,5)P_3 formation. The inability of immune complexes to cause increased intracellular levels of calcium was considered to be possibly related to inadequate formation of Ins(1,4,5)P_3. The data obtained from five separate experiments demonstrated that stimulation of neutrophils with 100 nM FMLP caused a 2.2-fold increase in the production of Ins(1,4,5)P_3 as compared with basal levels of Ins(1,4,5)P_3. In contrast, there was much less Ins(1,4,5)P_3 formation (1.4-fold increase) in neutrophils stimulated with immune complexes containing 60 μg antibody/ml (Fig. 4). The differences between Ins(1,4,5)P_3 levels in cells stimulated with FMLP or with immune complexes were statistically highly significant ($p < 0.006$). Thus, the inability of immune complexes to cause increased levels of $[Ca^{2+}]_i$ in neutrophils may at least in part be related to diminished formation of Ins(1,4,5)P_3.

Resistance of O_2^- responses to pertussis toxin pretreatment of neutrophils. Since it is well known that signal transduction responses of FMLP-stimulated neutrophils are sensitive to the inhibitory effects of pertussis toxin (14), the superoxide responses of pertussis toxin-treated or untreated neutrophils were measured after cell stimulation with FMLP (100 nM), immune complexes (containing 60 μg antibody/ml) or PMA (10 ng/ml). The results from five separate experiments, shown in Figure 5*A*, demonstrate the high degree of inhibition (89%) of the O_2^- responses to FMLP in cells pretreated with per-

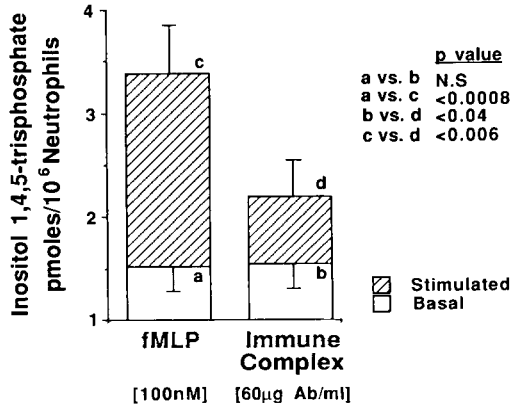

Figure 4. Peak Ins(1,4,5)P_3 level in 2×10^7 human neutrophils stimulated with 100 nM FMLP or immune complexes containing 60 μg antibody/ml. Peak concentrations of Ins(1,4,5)P_3 produced by FMLP occurred at 10 to 20 s; in the case of immune complexes this occurred 30 to 40 s after the addition of complexes (data not shown). Results are presented as $\bar{x}$ ± SEM of five separate experiments, each involving stimulation with FMLP or immune complexes. Companion cell samples incubated at 37°C under the same conditions but in the absence of agonist are also shown (*open bars*). Statistical comparisons between groups are indicated.

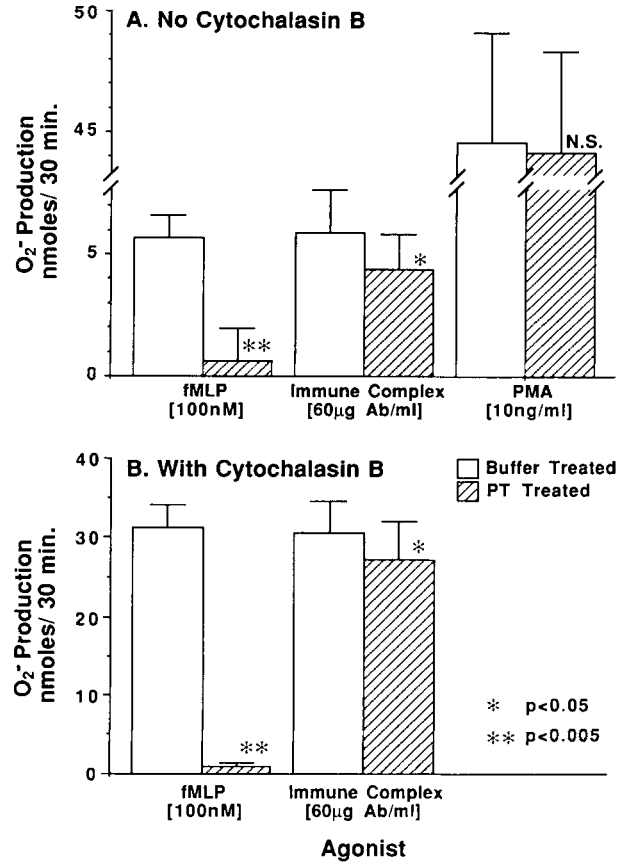

Figure 5. O_2^- production (nmol/30 min/2×10^6 cells) in neutrophils stimulated with 100 nM FMLP, immune complexes containing 60 μg antibody/ml, or PMA (10 ng/ml) in the absence (*A*) or presence of 2.5 μg/ml cytochalasin B (*B*). In each case companion sets of cells that had been pretreated with pertussis toxin were employed. Results represent the $\bar{x}$ ± SEM from five separate experiments.

tussis toxin. In contrast, the responses to immune complexes in pertussis toxin-treated cells were only slightly attenuated (by 26%, $p < 0.05$). As anticipated, the PMA-induced O_2^- response was totally resistant to the effects of pertussis toxin treatment. Because the presence of cytochalasin B amplifies the O_2^- response of the neutrophil, we evaluated neutrophil responses (in four separate experiments) to FMLP and immune complexes in the presence of cytochalasin B (2.5 μg/ml). Again, the variable was pertussin toxin pretreatment of cells. As is evident in Figure 5*B*, the O_2^- response to FMLP was almost totally abolished (>90% inhibition) in pertussis toxin-treated cells whereas there was very little inhibition (<11%) of the O_2^- response to immune complexes. These data suggest that the O_2^- response to immune complexes is largely resistant to the effects of pertussis toxin, in contrast to the highly sensitive nature of the FMLP-induced response.

Effects of antibodies to FcγR on the O_2^- responses to immune complexes. Because at least three distinct FcγR subclasses exist on human neutrophils (reviewed in Reference 28), mAb to each receptor were used to evaluate effects on O_2^- production after exposure to immune complexes. For the experiments shown in Figure 6, neutrophils were pretreated with 4 μg/ml of the intact (*A*) or the Fab/F(ab')$_2$ fragments (*B* and *C*) of the mAb 32.2 (anti-FcγRI), IV.3 (anti-FcγRII), or 3G8 (anti-FcγRIII). Antibody-treated cells were then diluted (without washing) and stimulated with immune complexes. Under the conditions employed, antibody addition did not affect the O_2^- response to FMLP (data not shown). As shown in Figure 6*A*, pretreatment of neutrophils with intact mAb to FcγRII or FcγRIII completely blocked O_2^- formation after addition of immune complexes ($n = 4$). Anti-FcγRI antibodies were without an effect on O_2^- production. Parallel experiments were performed with the Fab or F(ab')$_2$ fragments of antibodies to FcγR (Fig. 6*B* and 6*C*). The F(ab')$_2$ fragment of 3G8 reduced the immune complex-stimulated production of O_2^- by 47%, but similar treatment with Fab IV.3 or F(ab')$_2$ 32.2 had no significant effect (Fig. 6*B*). The pretreatment of cells with the combination of 32.2, IV.3, and 3G8 was no different from the effect of 3G8 alone. To improve the blocking by the Fab or F(ab')$_2$ fragments, cells pretreated with these antibody fragments were pelleted, resuspended, and then exposed to 4 μg/ml polyclonal F(ab')$_2$ fragment of goat IgG specific for mouse IgG (Fig. 6*C*). Exposure to the second antibody improved the effectiveness of blocking by the IV.3 Fab fragment, resulting in a 48% reduction in the immune complex superoxide response. This inhibition was comparable to the 55% reduction after 3G8 (F(ab')$_2$)/goat F(ab')$_2$ anti-mouse pretreatment. Under these conditions, cells pretreated with 32.2 antibody still failed to show a blocking effect. These studies indicated that unrestricted access to both FcγRII and FcγRIII is necessary for the optimal O_2^- response of human neutrophils to immune complexes.

FcγR-specific stimulation of the O_2^- responses. The experiments described above suggest that occupancy of both FcγRII and FcγRIII receptors is necessary for optimal O_2^- production to immune complexes. It has been suggested that FcγRIII may not participate in direct stimulation of the neutrophils but serves to modulate the FcγRII (36). In order to determine if cross-linking of

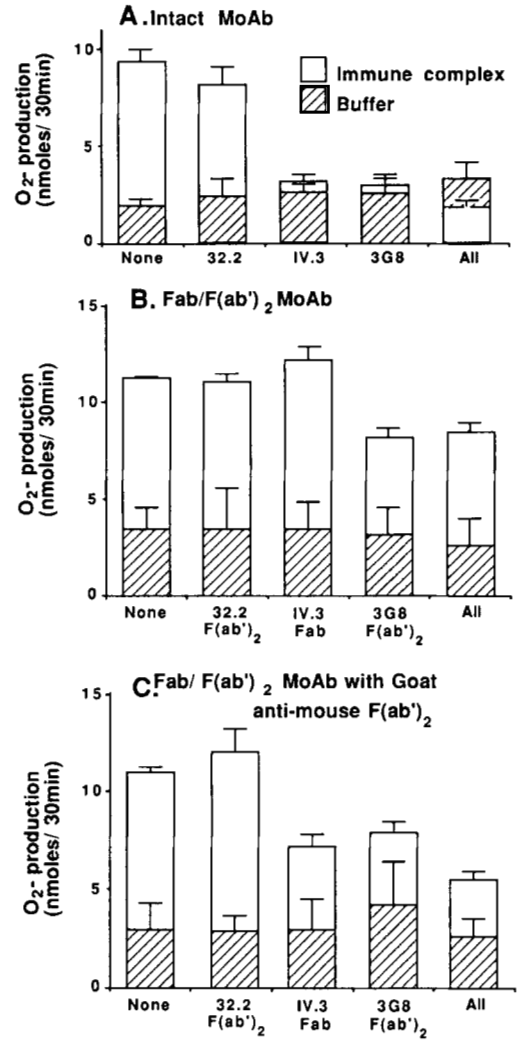

Figure 6. Inhibition of O_2^- production in immune complex-stimulated neutrophils by pretreatment with mAb 32.2 (anti-FcRγI), IV.3 (anti-FcRγII), or 3G8 (anti-FcRγIII). Neutrophils were preincubated with intact mAb (4 μg/ml) (*A*), Fab or F(ab')$_2$ fragments (4 μg/ml) (*B*), or monoclonal fragments (4 μg/ml) with polyclonal goat F(ab')$_2$ fragments for mouse IgG (4 μg/ml) (*C*) before the addition of immune complexes (60 μg antibody/ml). Results represent $\bar{x} \pm$ SEM of four (frame *A*) and three experiments (*B* and *C*).

FcγRII or FcγRIII initiates the respiratory burst, studies employing FcγR stimulation were undertaken. The stimuli for these experiments were formalin-fixed *S. aureus* cell walls that were coated by intact mAb. The antibody binds to protein A of *S. aureus* through the Fc portion of the antibody, leaving the Ag-binding region free to cross-link surface Ag. Figure 7 represents the means of four separate experiments in which neutrophils were exposed to *S. aureus*-coated particles with the intact mAb MOPC 21, MOPC 141, IV.3, or 3G8. Neither the uncoated *S. aureus* nor the *S. aureus*-coated particles with the nonspecific mAb (MOPC 21 and MOPC 141) resulted in O_2^- production that was significantly different from O_2^- found in unstimulated cells incubated in buffer solution alone. However, both IV.3- or 3G8-coated particles stimulated the production of O_2^-, comparable with the response to immune complexes (60 μg antibody/ml). The combination of both antibodies was slightly better than either antibody alone ($p < 0.02$). These studies confirm the blocking experiments and indicate that cross-linking

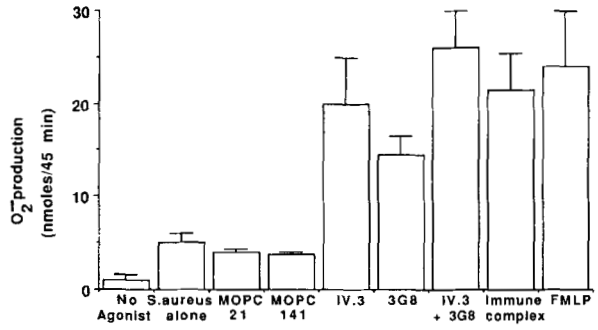

Figure 7. Direct FcγR-specific neutrophil stimulation of O_2^- response. Neutrophils (10^6) were stimulated with formalin-fixed *S. aureus* cell walls (0.2% (v/v)) untreated or coated with 1 μg of one of the following: MOPC 21 (mouse monoclonal IgG1), MOPC 141 (mouse monoclonal IgG2b), IV.3 (anti-FcRγII), 3G8 (anti-FCRIII), or IV.3 together with 3G8 (1 μg each). Data represent $\bar{x} \pm$ SEM of three experiments.

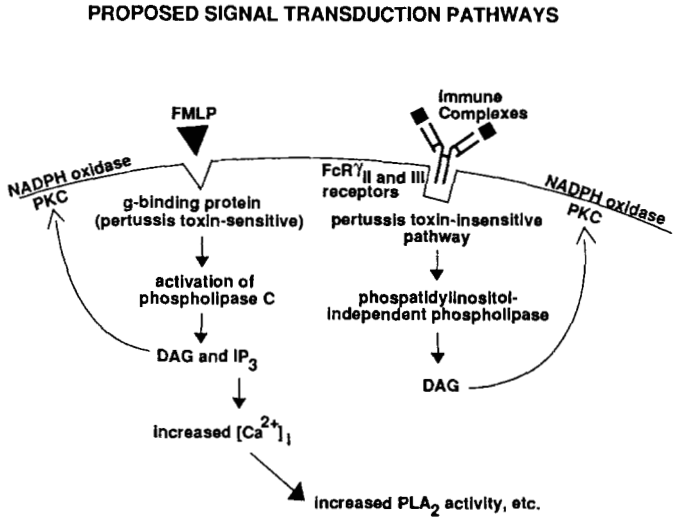

Figure 8. Proposed signal-transduction pathways in neutrophils stimulated with immune complexes or with FMLP.

of either FcγRII or FcγRIII receptors initiates O_2^- generation in neutrophils.

DISCUSSION

The data in this report describe the unexpected observation that the O_2^- response of human neutrophils to immune complexes is associated with little if any detectable increase in $[Ca^{2+}]_i$, transmembrane $[^{45}Ca^{2+}]$ flux, or production of Ins(1,4,5)P_3. The other striking feature of our observations is that, in contrast to the O_2^- response to FMLP, the O_2^- response to immune complexes is largely insensitive to the inhibitory effects of pretreatment of cells with pertussis toxin.

It is possible that signal transduction in human neutrophils employs two separate pathways, as depicted in Figure 8. The experiments using mAb to either FcγRII or FcγRIII suggest that either receptor can initiate the signal transduction cascade. Engagement of these two receptors activates the major pathway responsible for 70 to 80% of the O_2^- response that is resistant to the effects of pertussis toxin. Either there is a pertussis toxin-insensitive g-binding protein or a signal transduction pathway is involved that bypasses the requirement for a g-binding protein. From a functional standpoint, the latter would be similar to stimulation of neutrophils with phorbol ester (23, 33, 34). The second immune complex-related pathway leading to O_2^- generation contributes only in a minor way to the total amount of O_2^- produced and appears to involve signal transduction similar to the well described FMLP pathway, which involves the participation of a pertussis toxin-sensitive g-binding protein resulting in engagement of phospholipase C, the generation of Ins(1,4,5)P_3 and release of Ca^{2+} from intracellular storage sites (11). However, it is production of DAG that may be the common key event in the activation of the NADPH oxidase in neutrophils exposed to immune complexes or FMLP. In FMLP-stimulated cells activation of phosphoinositol-dependent phospholipase C leads to the production of DAG. There is increasing evidence that DAG may be derived from other substrates without the associated generation of Ins(1,4,5)P_3 (37–40).

The existence of two signal transduction pathways may explain the apparent contradiction between reports in which surface bound or aggregated IgG have produced calcium transients in leukocytes (34, 41–43) and other studies in which an increase in $[Ca^{2+}]_i$ has not occurred (34, 44, and this report). Similarly, this explanation may also account for the pertussis toxin sensitivity of the O_2^- response when FcγRII is cross-linked with antibody (31). The stimulus used in a given study (e.g., IgG-coated yeast or immune complexes) may preferentially activate one or the other of the two pathways described above. Support for this possibility comes from recent reports in which the sensitivity of the O_2^- response to pertussis toxin is dependent on the nature of aggregated IgG (45). In addition, it is also important to note that some FcγR-mediated responses to IgG subclasses may require some participation of a g-binding protein (46).

Acknowledgments. We wish to acknowledge the invaluable contribution of Kimberly Drake in preparation of this manuscript and Robin G. Kunkel for her assistance in preparation of figures.

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