Neutrophil Activation Through High-Affinity Fcγ Receptor Using a Monomeric Antibody With Unique Properties

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The high-affinity, type I Ig Fc receptor (FcγRI) for human IgG1, human IgG3, murine IgG2a, and murine IgG3 is highly expressed on monocytes, neutrophils (PMN) in certain disease states, and phagocytes treated with interferon-γ (IFN-γ). We studied the activation of the human PMN oxidative burst and stimulated fluid pinocytosis induced by three monoaonal antibodies (MoAbs) directed against FcγRI (CD64) to study the role of this receptor in Fc-mediated cellular activation. All three MoAbs were capable of triggering PMN activation from IFN-γ-treated PMN when cross-linked with goat antimurine Ig reagents. However, MoAb 197 alone demonstrated a concentration-dependent activation of the oxidative burst without the use of a second cross-linking antibody. The oxidative burst and stimulated fluid pinocytosis responses induced by monomeric MoAb 197 could be elicited only after the IFN-γ induction of approximately 8,000 FcγRI receptor equivalents and did not occur in freshly isolated or control-cultured PMN. Competitive blocking of Fc binding of MoAb 197 by human IgG or purified Fc fragments inhibited cellular activation. We believe the ability of MoAb 197 to activate these oxidative burst and fluid pinocytic responses was because of its murine IgG2a subclass, which allowed it to function as a trivalent anti-FcγRI antibody binding through the combination of its two Fab regions and the Fc domain. This study demonstrates that the cross-linking of CD64 can activate PMN oxidative and endocytic responses and supports a role for FcγRI in the human neutrophil inflammatory response.

Fcγ RECEPTOR-MEDIATED polymorphonuclear leukocyte (PMN) activation requires cross-linking of Fcγ receptors before cellular activation.1 This occurs as a physiologic defense mechanism when PMN contact opsonized bacteria, triggering phagocytosis and release of toxic cellular products. Pathologic conditions, such as serum sickness, glomerulonephritis, and vasculitis exist where circulating or deposited immune complexes trigger this same Fc-mediated cellular activation, causing detrimental consequences to the host.2 In vitro, this has been modeled with opsonized beads, aggregated IgG, and antigen-antibody immune complexes with little attention directed to the class of the Fc receptor involved. A better understanding of these cellular processes is a necessary prerequisite to a more rational therapeutic approach to the various immune complex-related diseases.

Three classes of human IgG Fc receptors (FcγRI, FcγRII, and FcγRIII) have been identified and defined through immunologic, physical, and functional characteristics.3 FcγRI (CD64) is the high-affinity Fc receptor for monomeric mouse IgG2a and IgG3 and human IgG1 and IgG3. FcγRII (CD32) and FcγRIII (CD16) are low-affinity Fc receptors for complexed IgG. At least one class of FcγR is expressed on monocytes, macrophages, PMN and their precursors, eosinophils, B cells, natural killer cells, and platelets.

PMN may express all three classes of FcγR. Resting PMN constitutively express FcγRII and FcγRIII at levels of 2 to 4 x 10⁴ and 1 to 2 x 10³ molecules per PMN, respectively. The expression of FcγRI, although minimal in resting PMN, is physiologically upregulated in patients with group A Streptococcal pharyngitis.4 In addition, the administration of interferon-γ (IFN-γ) induces the expression of FcγRI in vivo5 and in vitro.6-13 FcγRI has been shown to be a physiologically relevant cell surface receptor for neutrophil- and monocyte-mediated cytotoxicity10,14 and the activation of an oxidative burst in monocyte cell lines.15,16

The monoclonal antibodies (MoAbs) 197 (IgG2a), 32.2 (IgG1), and 22 (IgG1) identify FcγRII through different, non-Fc-binding domains of the receptor.14 MoAb 197 has the unique ability among these mouse MoAb to potentially function as a trivalent antibody as the two variable regions are directed against a non-Fc-binding epitope of FcγRI, and the Fc domain acts as a high-affinity ligand for the Fc-binding domain of FcγRI. This increased valency makes it an ideal candidate antibody for the study of FcγRI-mediated activation. The purpose of this study is to isolate and characterize the early stages of FcγRI-mediated activation using these anti-CD64 MoAbs in an attempt to understand the role of specific FcγR classes in Fcγ-mediated PMN activation.

MATERIALS AND METHODS

Reagents. Human recombinant IFN-γ was a gift from Genentech (South San Francisco, CA) and IFN-γ units were determined by a rapid, simple bioassay specific for human IFN-γ, the FcR-augmenting factor assay.7 Purified or supernatant anti-FcγRI MoAbs used in these studies were obtained from Mederex (Lebanon, NH) and included MoAb 197, MoAb 22, and MoAb 32.2. MoAb 197, also obtained from Mederex, an IgG1 murine MoAb that does not bind specifically to any human leukocyte surface antigen, was used as a control for nonspecific binding. FITC-conjugated goat antimouse F(ab')2 antibody preparations were obtained from Caltag Laboratories (South San Francisco, CA). All antibody preparations were azide free. Heat-aggregated human IgG was prepared in the standard fashion by incubating 25 mg/mL

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Submitted July 27, 1989; accepted October 5, 1990.

Supported in part by grants from the Upjohn Company (B.H.D.); American Heart Association, NH Affiliate (B.H.D.), National Cancer Institute (CA43476, DK33100, CA17223), and Friends of the Norris Cotton Cancer Center (W.L.A.).


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0606-4971/91/7703-0028$3.00/0
in phosphate-buffered saline (PBS) at 63°C for 10 minutes and washing free of soluble IgG with PBS. Purified Fc fragments of human IgG were obtained from Cappel (West Chester, PA).

Dichlorofluorescin diacetate and propidium iodide was obtained from Molecular Probes (Eugene, OR). RPMI 1640 and fetal bovine serum were from Hyclone (Logan, UT). Purified human IgG Fc fragments were obtained from Cappel (West Chester, PA). Fluorescein (FITC) standard latex beads were obtained from Flow Cytometry Standards (Research Triangle Park, NC). All other reagents were obtained from Sigma Chemical (St Louis, MO).

**PMN preparations.** PMN from normal healthy volunteers were used in all experiments. Following the institution-approved informed consent procedure, whole blood was drawn into a syringe containing preservative-free heparin. After sedimentation with high molecular weight dextran (2% final concentration), PMN were further purified using density gradient centrifugation with Ficoll-Hypaque. The remaining red blood cells were removed through brief hypotonic lysis. Purified PMN were resuspended to 2 × 10^6 cells/mL in RPMI 1640 supplemented with 10% fetal bovine serum and cultured for the indicated lengths of time at 37°C in 5% CO₂.

**Indirect immunofluorescence assay.** PMN were removed from culture media and incubated for 2 hours at 4°C with saturating concentrations of purified MoAb in the presence of 4.8 × 10⁻⁵ mol/L human IgG, which effectively blocks the Fc region of the MoAb from binding to the FcyRI. The cells were then washed three times with 1.0 mL PBS plus 2 mg/mL of bovine serum albumin (BSA) at 4°C, and counterstained with saturating concentrations of FITC-conjugated goat F(ab')₂, directed against mouse Ig (GAM-FITC). All samples were washed three times at 4°C and resuspended in 200 to 400 μL of PBS-BSA for fluorescent analysis on a Coulter Epics 741 flow cytometer (Hialeah, FL). Excitation was at 488 nm from an argon ion laser at 250 mW, and green fluorescence was measured using a 525-band pass filter. All samples were either examined promptly at 4°C or at a later time after fixation with 2% paraformaldehyde. Debris, aggregated PMN, and other cell types were identified by light scatter parameters and excluded from analysis. Fresh samples were washed with propidium iodide (2 μg/mL) in earlier experiments to further exclude nonviable PMN from analysis, but this was discontinued after determination that light scatter gating of the PMN population alone yielded equivalent results.

**Quantification of MoAb binding.** The relative number of receptors or antigens on the PMN cell surface was determined by the use of latex beads containing six different concentrations of fluorescein molecules per bead according to the method of LeBouteiller et al. The mean fluorescence intensity of each bead was determined and a standard regression curve of the measured fluorescence and actual molecules per bead was constructed. The mean number of FITC-labeled second goat-antimouse antibodies per cell was determined after subtraction for nonspecific antibody binding and autofluorescence. At saturating concentrations of a primary antibody, the results approximated the relative number of FcyRI molecules per PMN. In experiments where a subsaturating concentration of primary MoAb was used, the same calculation yielded a result that was proportional to the relative number of specific antibodies bound per cell.

**Oxidative burst assay.** The flow cytometric oxidative burst assay of Bass et al was performed with minor modifications. PMN were washed and adjusted to a concentration of 2 × 10⁶ in PBS containing 5 mmol/L glucose, 0.1% gelatin, and 2 mmol/L EDTA (PBS-gel). Extracellular calcium, magnesium, and azide were specifically excluded. Dichlorofluorescin diacetate (DCFDA) from a stock solution of 5 mmol/L in ethanol was incubated with the PMN suspension at a final concentration of 1 μmol/L at 37°C for 15 minutes. The suspension was subsequently divided into individual tubes containing the desired trigger substances or buffer (control). After 20 minutes of incubation, unless otherwise specified, the reaction was arrested by rapid dilution of the cell suspension with an equal volume of PBS-gel at 4°C and stored on ice before analysis. In previous studies, we have found the cell-associated fluorescence to be stable for at least 4 hours when handled in this manner. The trigger substances included phorbol myristate acetate (PMA) at a final concentration of 100 ng/mL, F-Met-Leu-Phe (FMLP) at a final concentration of 10⁻⁴ mol/L, heat-aggregated human IgG (HIG), or an MoAb preparation at a final concentration of 5 μg/mL, unless otherwise indicated. The final concentrations of the stock diluents, ethanol, and DMSO in the cell suspensions were less than 0.02% and 0.01%, respectively.

Instrument settings on the flow cytometer were similar to that described above, except lower PMT high voltages were used. The difference in fluorescent intensity between the stimulated and unstimulated control PMN, determined by a log-linear transformation of flow cytometric data, was proportional to the oxidative burst of the stimulated PMN. Debris, nonviable or aggregated PMN, and other cell types were identified by light scatter parameters and excluded from analysis. All samples were run at identical fluorescent photomultiplier tube settings and 5,000 PMN were collected per data point.

A comparison was made of the flow cytometric DCDFA oxidative burst assay to the spectrophotometric assay of superoxide anion production by measuring the superoxide dismutase inhibitable reduction of ferriytochrome C to ferrocyanochrome at 550 nm. Freshly isolated neutrophil preparations were split and assayed in parallel by the DCFDA and the cytochrome C methods for the degree of oxidative burst activity indicated by the two methods over a 15-minute period of PMN stimulation. Cells were activated with various concentrations of FMLP (10⁻⁵ to 10⁻⁷ mol/L), and 100 ng/mL PMA. Cytochalasin B (5 μg/mL) pretreatment of cells before FMLP stimulation was also studied to ensure a range of values of superoxide anion production. As shown in Fig 1, a linear correlation (R² = .89) between the two assays of oxidative burst was observed. Because the flow cytometric assay allows the
ability to exclude dead cells from analysis by using light scatter gating, it was deduced to be the superior assay for this study.

**PMN oxidative burst triggered through cross-linking second antibody.** PMN were washed and adjusted to a concentration of 1 to 2 × 10⁵ cells/mL in PBS-gel. DCFDA at a final concentration of 1 μM was incubated with the PMN suspension at 37°C for 15 minutes. The suspension was split to individual tubes containing the desired primary anti-FcγRI antibody or buffer (control) for 30 minutes at 4°C and then washed three times with PBS-gel at 4°C. Each PMN suspension was split into tubes containing GAM-FITC antibody at a final concentration of 5 μg/mL or buffer control before rewarming to 37°C. After a 20-minute incubation, the reaction was arrested by rapid dilution of the cell suspensions with equal volumes of PBS-gel at 4°C and stored on ice until analysis.

**Human IgG and Fc fragment inhibition of PMN activation.** The experiments involving human Ig (IgG) blocking of PMN oxidative burst were performed as described above except for the following modification. After the PMN were incubated with DCFDA for 15 minutes at 37°C, buffer (PBS-gel), Fc fragments, or varying concentrations of IgG in PBS-gel were added to the cell suspensions. Five minutes later, the appropriate activation stimuli were added and the remainder of the assay was run as stated above. All IgG solutions were ultracentrifuged at 160,000g for 30 minutes immediately before experimentation to remove aggregates from solution.

**Fluid pinocytosis (FP) activity.** Neutrophil-stimulated FP was measured on freshly isolated and cultured PMN using the previously described flow cytometric method for the quantitation of fluorescently labeled Molecular weight dextran fluid phase uptake. Fluid-phase endocytosis of 1 mg/mL FITC-dextran (molecular weight 70,000) was measured in FMLP, human immunoglobulin (HIG), anti-FcγRI antibody, and PMA-stimulated PMN preparations. Results are expressed as the increase in mean channel fluorescence of the stimulated PMN samples relative to unstimulated controls.

**Statistical analysis.** Unpaired t-testing and analysis of variance were used for comparison of experimental results. Linear regression and Pearson product-moment correlation were used to examine trends.

## Results

**Effect of in vitro IFN-γ treatment on PMN oxidative burst.** IFN-γ, used for the induction of FcγRI on PMN from healthy volunteers, may affect other functional characteristics of the PMN. For this reason, a baseline functional assessment of resting PMN and PMN cultured with or without IFN-γ was performed using FMLP and PMA as stimuli. The oxidative response of PMN was chosen as a parameter of activation because it is both a functional parameter of activation and a regulator of other PMN functions.

Both IFN-γ (100 U/mL) and in vitro culture caused changes in PMN survival or oxidative burst when triggered by non-Fc-mediated stimuli (Fig 2). IFN-γ resulted in a significant increase in PMN survival compared with control PMN after 48 hours of culture (42% ± 3.6% [standard error of the mean] v 30% ± 1.6%, P < .02). This difference was not significant when assessed after 24 hours of culture (92% ± 1.7% v 80% ± 7.3%, P < .16). Similarly, IFN-γ caused an enhancement of oxidative burst from PMN cultured for 48 hours when stimulated with FMLP (P < .02). The PMA-stimulated oxidative burst of PMN cultured for 24 or 48 hours and the FMLP-stimulated oxidative burst of PMN cultured for 24 hours were not modified by IFN-γ. The mean oxidative response from untreated, cultured PMN was greater than freshly isolated PMN after stimulation with FMLP (not significant) and PMA (P < .05) independent of IFN-γ treatment.

**FcγRI-mediated oxidative burst and FP.** PMN were examined for their capacity to respond to activation by anti-FcγRI antibodies. The PMN oxidative response induced by saturating concentrations (5 μg/mL) of MoAb 197, the IgG2a anti-FcγRI antibody, is shown in Fig 3. An oxidative response to MoAb 197 was observed only from PMN cultured for 24 hours were not modified by IFN-γ. The mean oxidative response from untreated, cultured PMN was greater than freshly isolated PMN after stimulation with FMLP (not significant) and PMA (P < .05) independent of IFN-γ treatment.

**Statistical analysis.** Unpaired t-testing and analysis of variance were used for comparison of experimental results. Linear regression and Pearson product-moment correlation were used to examine trends.

## Fig 2. Effect of culture and/or IFN-γ on PMN oxidative burst. PMN were freshly isolated or cultured for 24 or 48 hours in the presence or absence of IFN-γ, then examined for stimulated oxidative burst with FMLP (10⁻⁴ mol/L) and PMA (100 ng/mL). The stimulated response from IFN-γ-treated PMN was significantly different from control-cultured PMN only in the FMLP-stimulated, IFN-γ-treated PMN after 48 hours of culture (P < .02). Culture conditions alone enhanced the PMN-induced oxidative burst (P < .05). Values are the means of seven experiments ± SEM.

## Fig 3. MoAb 197-induced oxidative burst in IFN-γ-treated and control PMN. PMN were freshly isolated or cultured for 24 or 48 hours in the presence or absence of IFN-γ and were examined for stimulated oxidative burst with FMLP. IFN-γ-cultured PMN became progressively more responsive to MoAb 197 with time. Data represent the mean of four experiments ± SEM.
cultured in the presence of IFN-γ. MoAb 197 did not trigger an oxidative response in freshly isolated PMN or in cultured PMN (without IFN-γ), even though the latter had an enhanced oxidative response to FMLP and PMA. The viable PMN showed a unimodal distribution of increased fluorescence intensity, reflective of the oxidative burst, after stimulation by MoAb 197, suggesting the balanced participation of all PMN rather than activation of a minor subpopulation of cells.

The possibility that aggregates of MoAb 197 might contribute to the observed 197-induced oxidative burst was addressed by ultracentrifugation of MoAb 197 preparations at 160,000g for 30 minutes to remove putative aggregates. MoAb 197 handled in this manner was equally effective when compared with noncentrifuged MoAb 197 in stimulating an oxidative burst in IFN-γ-treated PMN, suggesting that large aggregates in the MoAb preparations do not contribute significantly to this particular PMN activation process.

MoAbs 32.2 and 22, non-IgG2a/3 anti-FcyRI mouse MoAbs, were unable to elicit an oxidative burst from PMN under the described conditions. However, all three anti-FcyRI antibodies triggered a comparable oxidative response in PMN cocultured with IFN-γ for 24 hours when cross-linked by a second goat antimouse F(ab′)2 antibody (Fig 4). Activation by second antibody cross-linking of the anti-FcyRI MoAbs 32.2, 22, and 197 also gave a unimodal population of increased DCF fluorescence relative to the unstimulated controls, indicating the activation of the entire PMN population. Second antibody cross-linking of MoAbs 32.2, 22, or 197 did not result in activation of freshly isolated PMN or PMN cultured in the absence of IFN-γ.

To better define FcyRI-mediated PMN activation, the 197-mediated oxidative burst was compared with the maximal activity induced by other PMN stimuli. In addition, stimulated FP a recently described event of PMN activation of uncertain physiologic significance,20,21 was studied as an alternate assay of PMN activation. The 197-mediated oxidative burst and FP was found to be induced by IFN-γ in a dose-dependent fashion after 24 hours in vitro culture conditions (Fig 5). Both assays showed the FcyRI-mediated PMN activation by 197 was of significantly less magnitude than FMLP (10−9 mol/L), aggregated IgG (1 mg/mL), or PMA (100 ng/mL) with cells treated for 24 hours with IFN-γ. The maximal oxidative burst induced by 197 in IFN-γ-treated PMN was 12% of that caused by maximal FMLP stimulation, 17% of the aggregated IgG activity, and less than 10% of the PMA activity. Similarly, the 197-induced FP activity under these conditions was 32% of the FMLP, 31% of the aggregated IgG, and less than 10% of the PMA activation levels of this endocytic response.

Relationship of FcyRI surface expression and 197-mediated burst. Quantification of the FcyRI surface expression and the 197-induced oxidative burst from PMN cultured with IFN-γ showed a linear increase over time (Fig 6). The FcyRI surface expression of freshly isolated PMN was found to be less than 2,000 receptor equivalents per cell. Treatment with IFN-γ resulted in an approximate linear increase in FcyRI expression at a rate of approximately 600 receptors per hour over the 48 hours of observation (R2 = .96). This modulation of FcyRI was dependent on IFN-γ and was not observed in buffer-treated control PMN.

The oxidative burst induced by MoAb 197 from PMN cocultured with IFN-γ also increased with time, increasing in parallel with the FcyRI expression (Fig 6). Regression analysis of the relative expression of FcyRI and the oxidative burst mediated by 197 activation in samples cultured for various periods with IFN-γ yielded an R2 value of .894. A threshold duration of 12 to 18 hours of culture with IFN-γ was required before a 197-induced PMN oxidative burst was clearly measured. The time required to overcome this threshold would, therefore, correspond to a minimal FcyRI surface expression of 6,000 to 10,000 receptor equivalents per PMN as a prerequisite for 197-mediated oxidative burst.

197 Dose response. MoAb 197 induced a dose-dependent activation of PMN cultured for 18 to 24 hours with IFN-γ when assayed for oxidative burst (Fig 7) and stimulated FP (results not shown). As the concentration of MoAb 197 was increased, the oxidative response also increased until a maximal stimulus was achieved. This plateau occurred at approximately the same concentration as receptor binding saturation of MoAb 197. The magnitude of the plateau varied with the duration and concentration of IFN-γ treatment as described in the previous section. Concentrations of MoAb 197 as high as 50 μg/mL had no further effect on the magnitude of the oxidative burst. In particular, no decrease in the oxidative burst and FP response was seen with a high dose of MoAb 197.

A correlation of the amount of MoAb 197 bound per PMN and the subsequent oxidative burst was performed (Fig 8). At low subsaturating concentrations of MoAb 197, there was no measurable generation of oxidative products.
CD64 NEUTROPHIL ACTIVATION

As the MoAb bound per PMN was increased, a threshold value was surpassed associated with cellular activation by 197. By regression analysis of the data from each experiment using only data points where a burst was measured, it was inferred that a minimum of 7,000 to 9,000 molecules of MoAb 197 were bound per PMN before detection of the PMN oxidative burst. This value is in accord with the above findings that induction of 6,000 to 10,000 FcγRI per PMN was required for a 197-induced oxidative burst.

MoAb 197 oxidative burst and FP kinetics. The oxidative burst and FP kinetics of PMN cultured for 24 hours with IFN-γ and stimulated by MoAb 197 differed significantly from the activity induced by FMLP and aggregated IgG in magnitude and rate (Fig 9). Both FMLP and aggregated IgG initiated nearly identical rapid initial “burst”-like production of oxidative metabolites with a plateau after 10 minutes. MoAb 197 triggered a slow, but continuous oxidative response. The kinetics of the FP response induced...
Relative molecules of MAE 197 bound per PMN

Fig 8. Correlation of the 197-induced oxidative burst and the relative molecules of MoAb 197 bound per PMN. Cultured PMN treated 24 hours with IFN-γ were incubated with varying concentrations of MoAb 197, and measured in parallel for the resulting oxidative burst and the number of molecules of MoAb 197 bound per PMN in three separate experiments. Linear regression lines, calculated only from those data points demonstrating a measurable oxidative burst, allow an estimate of the amount of bound MoAb 197 required to induce an oxidative burst.

by all three stimuli was similar in pattern with a maximal activity evident at 5 minutes, resembling that previously published for FMLP stimulation. All three modes of PMN activation showed a decrease in the sustained level of FP, although 197 activation showed a proportionally higher level of sustained activity relative to the maximal activity seen at 5 minutes poststimulation. Hence, as seen with the oxidative burst response, the 197-mediated FP response is more sustained over time relative to HIG and FMLP activation.

Blocking of the Fc interaction of MoAb 197 inhibits the oxidative response. As the Fc fragment of MoAb 197 has been shown to serve as a high-affinity ligand for FcγRI on monocytes, we postulated a similar mechanism on neutrophils. If the interaction between the Fc fragment of MoAb 197 and FcγRI were integral to PMN activation, then interruption of this interaction should inhibit the PMN oxidative response induced by MoAb 197 without altering the total number of MoAbs bound per PMN. This hypothesis was tested by preincubating IFN-γ cultured PMN with varying concentrations of human IgG or purified human Fc fragments before stimulation by MoAb 197. Because human IgG and Fc fragments have the capacity to compete with MoAb 197 at the Fc-binding domain of FcγRI, it would inhibit only Fc binding of MoAb 197 and not its Fab binding, which identifies an epitope distinct from the Fc-binding domain of FcγRI. Both HIG and Fc fragments caused a dose-dependent inhibition of the 197-induced oxidative burst (Fig 10). However, greater inhibition was achieved with Fc fragments with nearly complete blocking of the 197-induced oxidative burst at levels of 200 μg/mL of Fc fragment. The PMN oxidative burst triggered by FMLP or PMA was not inhibited by preincubation with HIG or Fc fragments. In parallel, we performed an indirect immunofluorescence assay quantifying the relative number of molecules of MoAb 197 bound per PMN, which showed that preincubation with HIG caused no quantitative difference in MoAb 197 binding. This implied that the observed graded inhibition of the 197-induced burst caused by HIG was not due to an absolute reduction in the amount of MoAb 197 bound per PMN, but rather to a graded qualitative difference in the Fc binding of MoAb 197 imposed by the HIG.

DISCUSSION

This study serves to extend previous observations that PMN can be induced to upregulate the surface expression

Fig 9. PMN oxidative burst and stimulated FP kinetics after stimulation with MoAb 197 (5 μg/mL), FMLP (10⁻⁶ mol/L), and aggregated IgG (1 mg/mL). PMN were cultured with IFN-γ for 24 hours, then examined for oxidative burst (B) and stimulated FP (A) activity relative to unstimulated controls at varying times after stimulation. Data represent the mean of five experiments ± SEM.
of FcγRI (CD64) by demonstrating the parallel activation of PMN functional responses mediated through this class of receptor. The degree of PMN activation induced through this FcγR receptor class with short-term IFN-γ treatment, as measured by oxidative burst and stimulated fluid pinocytosis, is not of the same magnitude as elicited through physiologic agents (FMLP, immune complexes) or direct pharmacologic activation of protein kinase C by PMA. However, the finding that the magnitude of these PMN functional responses parallels the surface expression of CD64 argues for physiologic significance to the PMN ability to modulate expression of this class of IgG receptor. Based on previous evidence that FcγRI-mediated antibody-dependent cytotoxicity can be demonstrated by IFN-γ-treated PMN and our demonstration of FcγRI-mediated oxidative burst in similarly treated cells, one could further speculate a role for CD64-mediated PMN activation in tissue injury or tumor cytotoxicity. As suggested by Vaickus et al.⁵ the in vitro demonstration of IFN-γ modulation of FcγRI receptors and subsequent PMN functional responses triggered through these receptors by MAB provides a scientific rationale for clinical trials combining FcγRI binding MoAb preparations and IFN-γ therapy.

Our findings further show that monomeric MoAb 197 directly activates a PMN oxidative burst and FP through FcγRI without the use of a second cross-linking agent. This property is unique among anti-FcγRI antibodies. The Fc fragment of MoAb 197 is distinguished from those of other anti-FcγRI antibodies by its IgG2a subclass, which confers on it the property of an affinity ligand for FcγRI. Our demonstration of inhibition of the 197-mediated oxidative burst and FP by Fc fragments and human IgG without a reduction in the total binding of the MoAb to IFN-γ-treated PMN provides strong evidence that the unique property of this anti-CD64 MoAb relates to its ability to function as a trivalent antibody binding through two separate and discrete epitopes of FcγRI through both its Fab and Fc fragments. Recent studies with the monocytic cell line U937 have similarly demonstrated direct activation by MoAb 197 of the oxidative burst, as measured by the cytochrome C reduction assay. The present study indicates that the same mechanism of MoAb 197-mediated cellular activation reported in mononuclear cells can be detected in neutrophils under conditions inducing sufficient FcγRI expression on the cell surface.

The activation of PMN by MoAb 197 or cross-linked anti-CD64 MoAbs is specific for PMN bearing a threshold level of FcγRI expression. Resting PMN with minimal expression of FcγRI fail to respond to anti-FcγRI MoAb as an isolated stimulus. Our findings of detecting 197-mediated PMN responses through FcγRI following low levels of IFN-γ (1.0 U/mL) provides a functional correlation and corroborates the recent study by Buckle and Hogg.¹³ reporting that human PMN FcγRI expression is extremely sensitive to IFN-γ. The minimum FcγRI surface expression necessary for activation is estimated at 8,000 FcγRI second antibody equivalents per PMN, a value similar to the minimum required number of MoAb 197 molecules bound per PMN for 197 activation when the surface expression of FcγRI is in excess. As the cell surface expression of FcγRI is increased above this threshold, the oxidative response to MoAb 197 also increased. Our results indicate that cross-linked couplets of FcγRI would also appear to be an inadequate stimulus for PMN activation, because MoAbs 32.2 and 22, IgG1 anti-FcγRI antibodies appear to be a second, possibly coincident, physiologic response to tissue injury or infection. Disease states that induce high endogenous production of IFN-γ would be expected to result in circulating PMN with high expression of CD64 and the capacity to become activated through this receptor. Based on previous evidence that the numbers of complement CR 1 and CR 3 on peripheral blood PMN, led us to postulate that upregulation of this receptor is involved in the pathophysiology of the acute inflammatory process. Just as PMN can upregulate the numbers of complement CR 1 and CR 3 receptors, the increased surface expression of FcγRI levels of IFN-γ (1.0 U/mL) provides a functional correlation and corroborates the recent study by Buckle and Hogg.¹³ reporting that human PMN FcγRI expression is extremely sensitive to IFN-γ. The minimum FcγRI surface expression necessary for activation is estimated at 8,000 FcγRI second antibody equivalents per PMN, a value similar to the minimum required number of MoAb 197 molecules bound per PMN for 197 activation when the surface expression of FcγRI is in excess. As the cell surface expression of FcγRI is increased above this threshold, the oxidative response to MoAb 197 also increased. Our results indicate that cross-linked couplets of FcγRI would also appear to be an inadequate stimulus for PMN activation, because MoAbs 32.2 and 22, IgG1 anti-FcγRI antibodies appear to be a second, possibly coincident, physiologic response to tissue injury or infection. Disease states that induce high endogenous production of IFN-γ would be expected to result in circulating PMN with high expression of CD64 and the capacity to become activated through this receptor. Based on previous evidence that the numbers of complement CR 1 and CR 3 on peripheral blood PMN, led us to postulate that upregulation of this receptor is involved in the pathophysiology of the acute inflammatory process. Just as PMN can upregulate the numbers of complement CR 1 and CR 3 receptors, the increased surface expression of FcγRI
capable of divalent binding through their Fab regions, fail to trigger activation. Therefore, the minimal required stimulus to signal FcγRI-mediated activation by MoAb 197 would appear to be the cross-linking of three or more FcγRI. It was not possible to further determine the actual number of required cross-links from these experiments because each FcγRI is capable of engaging two individual MoAb 197 molecules simultaneously, which may in turn link to multiple receptors creating a matrix of indefinite size, resulting in an activation signal. However, our findings that high levels of 197 (>10 μg/mL) do not cause a decrease in the 197-mediated oxidative burst suggest that large FcγRI complexes of much greater than three cross-linked receptors are not required for PMN activation. The time required for the process of intramembranous receptor migration, matrix formation, and sequential triggering may explain the slow, but sustained, kinetics of the oxidative response and FP in PMN activated by MoAb, in contrast to the “burst”-like response seen with classic ligand receptor activation of PMN by FMLP. Clearly, additional studies are required to dissect the intricate details of PMN activation through FcγRI.

The demonstration of an FcγRI-induced PMN oxidative burst or stimulated FP, absent in freshly isolated PMN, requires the combination of in vitro manipulation and treatment with IFN-γ. In these experiments and those of others, IFN-γ caused a marked enhancement of FcγRI surface expression seen after 12 hours of in vitro culture, yet only a modest influence on the overall stimulated respiratory burst assessed by FMLP, aggregated IgG, and PMA. Such in vitro culture conditions can enhance the stimulated oxidative burst independent of the FcγRI surface expression, which may be mediated by attachment to a plastic surface. Although in vitro manipulation does not appear to be the dominant factor in the PMN activation through FcγRI, its role should bear consideration through in vivo extension of this work. Demonstration of FcγRI-mediated PMN functional responses in patients with disease or iatrogenic states causing increased PMN expression of CD64 would further substantiate the physiologic importance of this receptor.

Our findings have established the properties of a monomeric MoAb with fortuitous biochemical properties that help to dissect the biology of cellular activation through FcγRI. Furthermore, our demonstration of FcγRI-mediated activation of the PMN oxidative burst and stimulated FP responses has extended the list of effector functions triggered through this FcγR subclass. Our results and those of others indicate that similar to the monocyte, which constitutively expresses FcγRI, the PMN CD64 Fe receptor subclass is able to mediate the activation of antibody-dependent cytotoxicity, and stimulate endocytosis, NADPH oxidase activity, or oxidative burst. Cellular responses mediated by the other Fc receptor subclasses on neutrophils, FcγRII (CD32) and FcγRIII (CD16), have only been partially elucidated. Both FcγRII and FcγRIII appear able to mediate lysosomal granule enzyme release. FcγRII has been shown repeatedly to be able to trigger a PMN oxidative burst using a variety of assay systems. However, the role of FcγRIII in activation of the NADPH oxidase system is unresolved. Our studies indicate a role for CD16 in this PMN response, but other groups have found no link between this FcγR subclass and the oxidative burst. The PMN phagocytic response has been reported to be mediated via FcγRIII for bacteria and concanavalin A-coated erythrocytes and via FcγRII for immune complex ingestion. Additional studies are required to more clearly define the functional role of the various classes of Fcγ receptor in neutrophils, but immunologic reagents such as MoAb 197 should prove to be a useful tool for such work.

ACKNOWLEDGMENT

We acknowledge the cell culture expertise of Dr Olive Petengill, the technical assistance of Rochelle Roscoe, and the secretarial assistance of Lisa Dupuis.

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