CR3 (Mac-1, $\alpha_M\beta_2$, CD11b/CD18) and FcγRIII Cooperate in Generation of a Neutrophil Respiratory Burst: Requirement for FcγRII and Tyrosine Phosphorylation

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Abstract. Cooperation among plasma membrane receptors in activating signal transduction cascades is not well understood. For almost 20 years, it has been clear that when a particulate foreign body is opsonized with complement as well as IgG, the efficiency of IgG effector functions is markedly enhanced. However, the molecular mechanisms involved in cooperation between IgG Fc receptors and complement receptors have not been elucidated. In this work, we show that when human neutrophils (PMN) are plated on a surface coated with both anti-CR3 and anti-FcγRIII antibodies, the respiratory burst which occurs is equivalent to that stimulated by anti-FcγRII. The CR3 ligand iC3b is as effective as anti-CR3 for cooperating with anti-FcγRIII in generation of a respiratory burst. The synergy between CR3 and FcγRIII for activating the NADPH oxidase is abolished by Fab of anti-FcγRII. Nonetheless, the observed synergy is not an artifact of unintended FcγRII ligation, since (a) only this combination of antibodies works to generate H_2O_2; (b) coating plates with either of the antibodies alone cannot activate the respiratory burst at any dose; (c) LAD (CR3 deficient) cells, which are perfectly competent to mount a respiratory burst when FcγRII is engaged, are incapable of activating the respiratory burst when adherent to wells coated with anti-FcγRIII and anti-CR3; (d) direct engagement of FcγRII activates the respiratory burst by a pathway pharmacologically distinguishable from the synergistic respiratory burst. FcγRIII/CR3 synergy is abolished by cytochalasin B and herbimicin, suggesting that both the actin cytoskeleton and tyrosine phosphorylation are necessary for activation of the synergistic respiratory burst. Further analysis shows that CR3 and FcγRIII have distinct roles in activation of this FcγRII-dependent assembly of the NADPH oxidase. Ligation of CR3 is sufficient to lead to FcγRII association with the actin cytoskeleton on the adherent PMN surface. Coligation of FcγRIII is required for tyrosine phosphorylation of FcγRII. These data are consistent with a model in which phosphorylation of FcγRII or a closely associated substrate initiates activation of a signal transduction pathway leading to oxidase assembly. These are the first data to demonstrate a molecular mechanism for synergy between IgG Fc and complement receptors in activation of phagocyte effector functions.

S INCE the work of Ehlenberger and Nussenzweig almost 20 years ago (17), it has been clear that when a particulate foreign body is opsonized with complement as well as IgG, the efficiency of IgG effector functions is markedly enhanced. The Ehlenberger-Nussenzweig hypothesis was that the role for complement interaction with complement receptors was to increase the efficiency of presentation of IgG to phagocyte Fc receptors. The implication of this hypothesis was that complement receptors did not contribute to the signal transduction which led to phagocytosis, degranulation, respiratory burst, and leukotriene production which occurred upon Fc receptor ligation. The lack of a role for complement receptor signaling in these functions has been supported by several studies (1, 42). However, recent studies have suggested that complement receptors may have a direct role in signal transduction in some phagocyte Fc receptor-mediated functions (10, 22, 25, 38). Biophysical studies from Petty's laboratory have shown that complement receptor 3 (CR3),[1] a member of the β_2 integrin family, cocaps with FcγRIII, the phosphoinositol glycan-

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1. *Abbreviations used in this paper:* CR3, complement receptor 3; DFP, diisopropylfluorophosphate; DOC, deoxycholic acid; IAP, integrin-associated protein; KRPG, Krebs buffer with glucose; PIG, phosphoinositol glycan; PBS Tween, phosphate buffered saline with 0.5% Tween 20; PMN, polymorphonuclear leukocytes; RM, reaction mixture.

linked Fc receptor on polymorphonuclear leukocytes (PMN), and that this cocapping can be inhibited by N-acetyl-d-glucosamine (43). From these data, Petty has hypothesized a direct physical interaction between FcγRIII and CR3 (38).

Together, these experiments suggest that when CR3 is ligated alone, signal transduction pathways may not be activated, but it has an important and necessary, but unexplained, role in signal transduction resulting from Fc receptor ligation. Despite these advances, direct tests of complement receptor-dependent signal transduction have been lacking. PMN express several complement receptors which recognize similar or identical ligands and also express at least two Fc receptors with similar ligand specificity. The resultant complexity of ligand-receptor interactions has made dissection of collaboration in signal transduction extremely difficult. Recently, an assay system using PMN adhesion to specific monoclonal antibodies has been developed which allows dissection of signal transduction phenomena (4, 44). In this work, we have used this assay system to understand Fc receptor-complement receptor collaboration in generation of a respiratory burst. We have found that the combination of anti-FcγRIII and anti-CR3 (or the CR3 ligand iC3b) leads to activation of a PMN respiratory burst, while neither antibody alone, nor iC3b alone, can do so. Anti-FcγRII, on the other hand, is competent to stimulate a respiratory burst on its own. Surprisingly, the respiratory burst stimulated by synergy between FcγRIII and CR3 is completely inhibited by Fab of anti-FcγRII. The synergistic respiratory burst is abrogated by cytochalasin B and by herbimicin, an inhibitor of tyrosine phosphorylation. Further analysis reveals that ligation of CR3 is sufficient to cause FcγRII association with the actin cytoskeleton, but that FcγRII is phosphorylated on tyrosine only when both CR3 and FcγRIII are ligated. These data are the first to provide a mechanism for cooperation between Fc receptors and complement receptors in phagocyte effector functions. We hypothesize that FcγRIII and CR3 have distinct roles in this collaboration, that ligation of CR3 signals an association of FcγRII with the actin cytoskeleton, and ligation of FcγRIII on the same adherent surface leads to tyrosine kinase activity in proximity with FcγRII. We suggest that this two-receptor-dependent coordination off enzyme and substrate initiates a pathway for assembly of the NADPH oxidase.

Materials and Methods

Monoclonal Antibodies

The following mAbs were used in these studies: IB4 (anti-CD18) (41); 3D9 (anti-CD 35) (33); W6/32 (anti-HLA) (2); B6H12 and 2D3 (anti-IAP, CD47) (11, 24); 3G8 (anti-CD16) (20), KuFc79 (40), and IV.3 (anti-CD32) (31). IB4, 3D9, 3G8, Ku79Fc, and IV.3 IgG were purified from ascites using octanoic acid as described (22). W6/32, B6H12, and 2D3 IgG were prepared using an Amicon Bioreactor (Amicon Inc., Danvers, MA) according to manufacturer's instructions. SDS-PAGE of all purified IgG preparations showed them to be >90% IgG. 4G10 (anti-phosphotyrosine, IgG2b) was from UBI (Lake Placid, NY). Fab fragments of W6/32, IV.3 and OKM1, F(ab')₂ fragments of 3G8 and 2D3 were prepared as described (34). FITC-conjugated antibody fragments were prepared as described (43). Immunoprecipitation studies have shown that there is no cross-reactivity between anti-FcγRII mAb and FcγRIII, or between anti-FcγRIII mAb and FcγRII (19, 31, 40).

Buffers and Other Reagents

Phosphate buffered saline was from Biowhittaker, Walkersville, MD. Krebs-Ringer buffer (KRPG) was 145 mM NaCl, 4.86 mM KCl, 1.22 mM MgSO₄, 5.7 mM Na₂HPO₄, 0.54 mM CaCl₂, and 5.5 mM glucose, pH 7.4. Reaction mixture (RM) consisted of 37.5 μM scopoletin, 1.25 mM NaN₃, 1.25 U/ml HPO in KRPG. Other reagents were obtained from standard sources as previously described (44).

PMN Isolation

Human PMN were isolated by dextran sedimentation and Ficoll-Hypaque density centrifugation as described (43). Normal human PMN express ~10–30 × 10³ FcγRII, 150–300 × 10³ FcγRIII, and 150 × 10³ CR3 (3, 23, 27, 39). PMN were obtained from two β₂ integrin-deficient (leukocyte adhesion deficient, LAD) patients followed at Baylor College of Medicine. Both patients are characterized as having the complete deficiency phenotype. Expression of FcγRII and FcγRIII on these patients' cells was normal, while CR3 was undetectable (not shown). The patient blood and a normal control were transported and the PMN prepared as described (25).

Preparation of Coated Plates

96-well tissue culture plates (Costar Corp., Cambridge, MA) were coated with protein A, and then mAb IgG essentially according to the method of Berton et al. (4), with modifications as described (44). When anti-CR3 OKM1 and anti-FcγRIII were combined with each other or other mAbs, 10 μg/ml of OKM1 and 20 μg/ml of 3G8 were used unless otherwise indicated. This combination of anti-CR3 and anti-FcγRIII led to an optimal respiratory burst. When these antibodies and other mAbs were used individually to coat plates, 30 μg/ml of IgG were added. To prepare surfaces coated with iC3b and antibody, 50 μg/ml each of protein A and human IgM were added in PBS to wells activated with poly-L-lysine and glutaraldehyde. After 4 h incubation at room temperature, the plates were blocked with casein, incubated with 20 μg/ml mAb or buffer as a control, and with 10% heat-inactivated FCS as described (4, 44). Finally, the plates were incubated with 5% fresh human serum in veronal-buffered saline with 1 mM Mg², 0.15 mM Ca², and 0.1% gelatin at 37°C for 90 min to deposit complement. The deposition of C3 fragments upon serum incubation with the IgM-coated plates was verified by ELISA with rabbit anti-human C3. The wells coated with human IgM alone or combined with protein A plus anti-FcγRIII 3G8 demonstrated similar C3 deposition, while surfaces coated with protein A alone or with 3G8 (anti-FcγRIII) did not activate C3 deposition (data not shown).

H₂O₂ Assay

The microwell H₂O₂ assay was adapted from the method of De la Harpe and Nathan (16), as modified by Berton et al. (4). Data were collected and analyzed exactly as previously described (44). In each experiment, data were averaged from triplicate wells, which generally varied from each other by ≤10%. For surfaces coated with human C3, the assay was performed in the presence of 5 μg/ml of anti-CR1 mAb 3D9, which is sufficient to block ligand binding to CR1, the C3b receptor of PMN (9). Unless otherwise stated, H₂O₂ accumulation was measured after 60 min of PMN incubation in wells.

Pretreatment of PMN

PMN at 2.5 × 10⁶ cells/ml in KRPG were pretreated with 5 μg/ml of Fab or 2.5 μg/ml of F(ab')₂ of various mAb at 4°C for 15 min. Without washing, PMN were added to antibody-coated plates containing RM (44). For treatment with pharmacologic agents, PMN were preincubated with herbimicin at 10 μg/ml or H-7 at 300 nM at 37°C for 15 min, and then added to RM containing the same concentration of the indicated agent. To pretreat PMN with pertussis toxin, cells in KRPG were incubated with 2 μg/ml of pertussis toxin at 37°C for 45 min. In experiments examining the effects of cytochalasins, PMN were pretreated with 2.5 μg/ml cytochalasin B or D and added to RM containing no additional drug. In all experiments, control PMN were incubated with an identical concentration of nonaqueous diluent as the PMN receiving any drug.

Immunofluorescence

4-well chamber slides (Nunc, Naperville, IL) were coated with mAbs as de-

scribed above for the 96 well plates. 2.5×10^5 PMN in KRPG were allowed to adhere to mAb-coated chamber slides at 37°C for 30 min, and the nonadherent cells were removed by briefly washing with PBS. The adherent cells were extracted with 0.5% Triton X-100 in extraction buffer (10 mM Hepes, pH 7.2, 300 mM sucrose, 100 mM KCl, 3 mM $MgCl_2$, 10 mM EGTA, 1 mM PMSF, and 2 mM diisopropylfluorophosphate [DFP]) for 5 min, and rinsed with extraction buffer once, and then fixed with cold methanol on ice for 20 min. Under this extraction condition, there were no residual granules or nuclei in the Triton-insoluble PMN ghosts visible under the phase contrast microscope. After rehydration in PBS, the slides were incubated with 0.5% BSA and 0.5% gelatin in PBS at room temperature for 30 min, and then with 10 μg/ml FITC-conjugated IV.3 (anti-FcγRII) Fab or W6/32 (anti-HLA) Fab as a control. After washing with PBS Tween, slides were mounted in 90% glycerol in PBS with 1 mg/ml OPD, and examined with a Nikon epifluorescence microscope with excitation of 485/30 nm and emission of 530/30 nm. To quantitate FcγRII fluorescence, 2.5×10^6 PMN were allowed to adhere to wells of 24-well plates coated with mAb as above, and then subjected to the same extraction and staining procedures. The immunofluorescence was quantitated using a Cytofluor 2300 plate reader (Millipore, Bedford, MA). In each experiment, the fluorescence of triplicate wells, which generally varied by ≤10%, were averaged. To combine data from different experiments, IV.3 Fab fluorescence on wells coated with other mAb was always compared to IV.3 Fab fluorescence in wells which had been coated with KuFc79, an anti-FcγRII mAb which does not compete with IV.3.

Immunoprecipitation

5×10^6 PMN in KRPG were allowed to adhere at 37°C for 30 min to 6-well tissue culture plates coated with various mAb as above. The adherent cells were extracted with detergent as described above, following which the plate was rinsed with 0.15 M sodium phosphate buffer containing 2 mM DFP and 0.2% Triton X-100 once. Proteins remaining adherent were radiolabeled in 500 μl of the same buffer by sequential addition of 50 μl of 22.5 mg/ml chloramine T and 5 μl of 100 mCi/ml ^{125}I. After three minutes, 50 μl of metabisulfite followed by 50 μl of 150 mM NaI were added to each well to quench the labeling reaction. After washing with the sodium phosphate buffer three times, the residual cytoskeletons were solubilized with solubilization buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1% DOC, 0.05% SDS, 10 μg/ml each of aprotinin, leupeptin and pepstatin A, 1 mM PMSF, 2 mM DFP) at 4°C for 60 min with shaking. The insoluble materials were removed by centrifugation in an Eppendorf 5415C microfuge (Brinkman Instruments, Westbury, NY), at top speed at 4°C for 10 min. The labeled lysate was then precleared with Sepharose-anti-murine IgG (mIg) at 4°C for 60 min. The precleared lysate was incubated with anti-FcγRII IV.3 IgG captured by Sepharose-anti-mIg at 4°C for 2 h. The Sepharose mixture was washed once with solubilization buffer, and then six times in the same buffer without DOC, SDS, and protease inhibitors, containing 5 mM KI. The Sepharose-bound radiolabeled proteins were analyzed by SDS-PAGE as described (12). To determine the effect of addition of Fab of anti-FcγRII IV.3 on association of FcγRII with the Triton-insoluble cytoskeleton, the same protocol was performed using PMN treated with 5 μg/ml of anti-FcγRII IV.3 Fab or with buffer during PMN adherence to antibody-coated plates. In these experiments, FcγRII was immunoprecipitated with KuFc79.

Tyrosine Phosphorylation

5×10^6 PMN in KRPG with 0.1 mM sodium vanadate were allowed to adhere to mAb-coated 6-well plates at 4°C for 10 min, and then at 37°C for 15 min. The nonadherent cells were removed by washing with cold PBS. The adherent cells were lysed with the cold solubilization buffer plus 1 mM sodium vanadate, 1 mM sodium pyrophosphate, 0.1 mM sodium molybdate, 100 μM phenylarsine oxide, 10 mM NaF. After immunoprecipitation and 10% SDS-PAGE, the immunoprecipitated proteins were transferred to polyvinylidine difluoride (Millipore, Bedford, MA). The membranes were incubated overnight in blocking buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 2% BSA, 0.5% NP-40), and then with 1 μg/ml anti-phosphotyrosine mAb 4G10 (UBI, Lake Placid, NY) in blocking buffer for 2 h at RT. The membranes were washed with the same buffer without BSA for four times, and incubated with HRP-conjugated anti-mouse IgG2b (Caltag Laboratories, South San Francisco, CA) at 1:1,000 dilution in blocking buffer at RT for 1 h. After three washes, the blots were developed using a chemiluminescence kit (Amersham Corp., Arlington Heights, IL).

Results

Costimulation of CR3 and FcγRIII with Surface Bound mAbs Activates the Respiratory Burst in PMN

Neutrophils express all three β_2 integrins, CR3, LFA-1, and p150,95. Although CR3 are the most abundant, ligation of CR3 does not trigger a respiratory burst, while ligation of either LFA-1 or p150,95 does (4). Similarly, although FcγRIII molecules are 10–15 times more abundant than FcγRII molecules on PMN, ligation of FcγRII, but not FcγRIII, initiates the respiratory burst (27, 28). Using mAb specific for FcγRII, FcγRIII, and CR3 to coat surfaces, we confirmed that anti-FcγRII, but not anti-FcγRIII or anti-CR3, could stimulate a PMN respiratory burst (Fig. 1 A). Increasing or decreasing the antibody concentration with which the plates were coated by up to 10-fold did not induce a PMN respiratory burst from PMN adherent to anti-CR3 or anti-FcγRIII. Surprisingly, when wells were coated with both anti-FcγRIII and anti-CR3, PMN produced H_2O_2 (Fig. 1 A). The effect of activating the respiratory burst was specific for engagement of this combination of receptors, since neither anti-FcγRIII nor anti-CR3 could stimulate a respiratory burst in combination with anti-HLA (Fig. 1 A) or in combination with anti-CR1 (not shown), both of which are prominent plasma membrane molecules of PMN. A second anti-CR3 mAb (10B5 [21]) also did not activate the respiratory burst when used alone, but did synergize with anti-FcγRIII (not shown). Activation of H_2O_2 production required that both anti-FcγRIII and anti-CR3 antibodies be adherent to the tissue culture dish. Addition of Fab or $F(ab')_2$ of either antibody in the fluid phase did not activate the respiratory burst in cells adherent to wells coated with the other antibody, even when the antibodies bound to the apical plasma membrane were cross-linked with $F(ab')_2$ anti-mouse IgG at a concentration sufficient to activate an increase in $[Ca^{+2}]_i$ from the 3G8 $F(ab')_2$ (Fig. 1 B) (35).

The dose response for this synergistic respiratory burst activation was biphasic for both antibodies (Fig. 2); all further experiments were done using wells coated with 20 μg/ml 3G8 (anti-FcγRIII) and 10 μg/ml OKM1 (anti-CR3), which led to optimal H_2O_2 production, or with 30 μg/ml of individual antibodies. The respiratory burst stimulated by the combination of anti-CR3 and anti-FcγRIII mAbs could be specifically inhibited by anti-CR3 Fab or anti-FcγRIII $F(ab')_2$ alone, but not by anti-HLA Fab (Fig. 3 A). This indicated that both CR3 and FcγRIII are directly involved in signal transduction for the respiratory burst. Surprisingly, the respiratory burst activated by synergy between CR3 and FcγRIII also was inhibited by anti-FcγRII Fab (Fig. 3 A). Inhibition of this synergistic activation of the respiratory burst was specific, since anti-FcγRII (IV.3) Fab did not inhibit H_2O_2 production by PMN adherent to IB4- (anti-β_2 [4]) or B6H12- (anti-IAP [44]) coated surfaces (Fig. 3 B). These results suggest that FcγRII is required for the respiratory burst induced by synergy between FcγRIII and CR3.

To determine whether natural ligand for CR3 would work similarly to antibody, PMN were adhered to microtiter wells coated with complement. H_2O_2 production was detected only when anti-FcγRIII also was present on the adherent surface (Fig. 4 A). Like the synergistic respiratory burst on antibody-coated surfaces, the respiratory burst generated
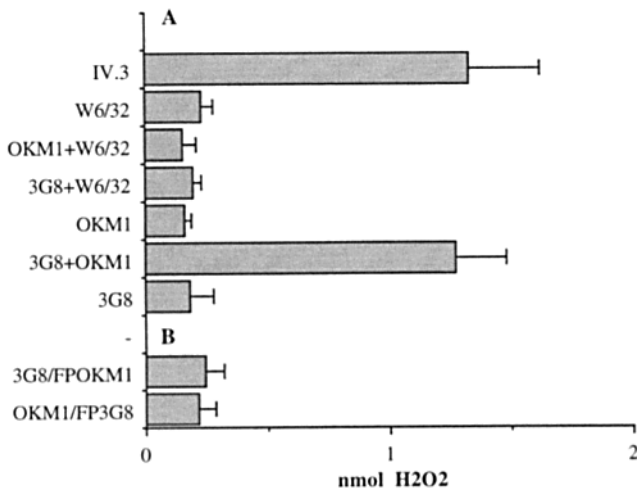
Figure 1. Activation of a PMN respiratory burst by adhesion to surface-bound mAb. The generation of H_2O_2 by normal PMN incubated in microwells coated with various mAbs alone and in combination is compared. PMN were incubated for 60 min on mAb-coated plates. IV.3 is anti-FcγRII, OKM1 is anti-CR3, 3G8 is anti-FcγRIII, and W6/32 is anti-HLA. Data are means $\pm$ SEM of three independent experiments, each performed in triplicate. In B, PMN were allowed to adhere to microwells coated with anti-CR3 or anti-FcγRIII alone. F(ab')$_2$ anti-FcγRIII was then added at 2.5 μg/ml to PMN on the anti-CR3-coated surface, and anti-CR3 Fab at 5 μg/ml to PMN on the anti-FcγRIII-coated surface. Cross-linking of antibody on the apical plasma membrane was achieved by addition of rabbit anti-mIg at 2.5 μg/ml. Data are means $\pm$ SEM of three independent experiments, each performed in triplicate. Anti-FcγRII can stimulate a respiratory burst when adherent to a surface, as can the combination of anti-CR3 and anti-FcγRIII. Both anti-CR3 and anti-FcγRIII must be surface-associated to synergize for stimulation of a respiratory burst.

from PMN adhesion to plates coated with anti-FcγRIII and complement was inhibited by anti-FcγRIII 3G8 F(ab)$_2$, by anti-CR3 OKM1 Fab, and by anti-FcγRII IV.3 Fab (Fig. 4 B). Thus, iC3b ligand and anti-CR3 antibody have the same ability to synergize with ligation of FcγRIII for signaling activation of the PMN respiratory burst and show the same inhibition by IV.3 Fab.

One possible explanation for the effect of anti-FcγRII Fab in this system is that FcγRII is engaged by Fc fragments of mAb on the adherent surface which have not been sufficiently blocked by the protein A on the plate. Engagement of FcγRII is a potent agonist for H_2O_2 generation (Fig. 1 A). Against this possibility are the observations that only one combination of antibodies is able to induce the respiratory burst and that neither of these antibodies alone can activate the respiratory burst at any dose; and that Fab or F(ab')$_2$ of either mAb can inhibit the respiratory burst, suggesting that both membrane ligands must be engaged. Unfortunately, coating wells with Fab or F(ab')$_2$ of mAb is ineffective at activating the respiratory burst in this experimental system ([4] and our unpublished results). To definitively rule out this artifact as the explanation for FcγRII involvement in the synergistic respiratory burst, we examined PMN of two LAD patients (Fig. 5). Although both patients made a normal respiratory burst on surfaces coated with anti-FcγRII, neither was able to generate H_2O_2 in response to surfaces coated with anti-
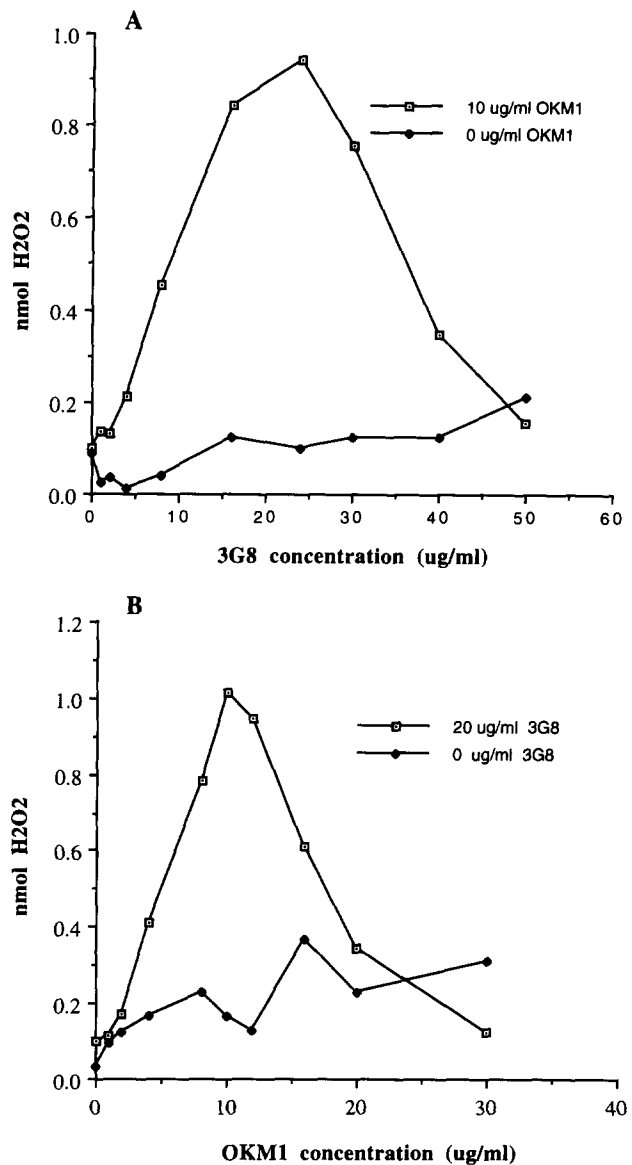
Figure 2. Dose dependence of anti-CR3 and anti-FcγRIII for respiratory burst activation. In A the dose response for 3G8 (anti-FcγRIII) in the presence ($\square$) or absence ($\blacklozenge$) of a constant amount of OKM1 (anti-CR3) is presented. A similar dose response for OKM1 in the presence ($\square$) or absence ($\blacklozenge$) of a constant amount of 3G8 is shown in B. All antibodies were surface-adherent. The data are from a single experiment performed in triplicate, representative of at least five experiments. Neither antibody alone was able to stimulate a respiratory burst at any concentration tested. The optimal concentrations of anti-CR3 and anti-FcγRIII for coating plates to achieve a maximal synergistic respiratory burst are 10 μg/ml and 20 μg/ml, respectively.

FcγRIII and anti-CR3. Thus, CR3 must be expressed on the PMN to activate the synergistic respiratory burst. If the synergistic effect were merely the result of FcγRII engagement by exposed Fc fragments on the plate, LAD cells should respond normally, even if FcγRII/FcγRIII cooperation (36) were required. This rules out involvement of FcγRII in the synergistic respiratory burst by receptor engagement with known ligands on the activating surface.
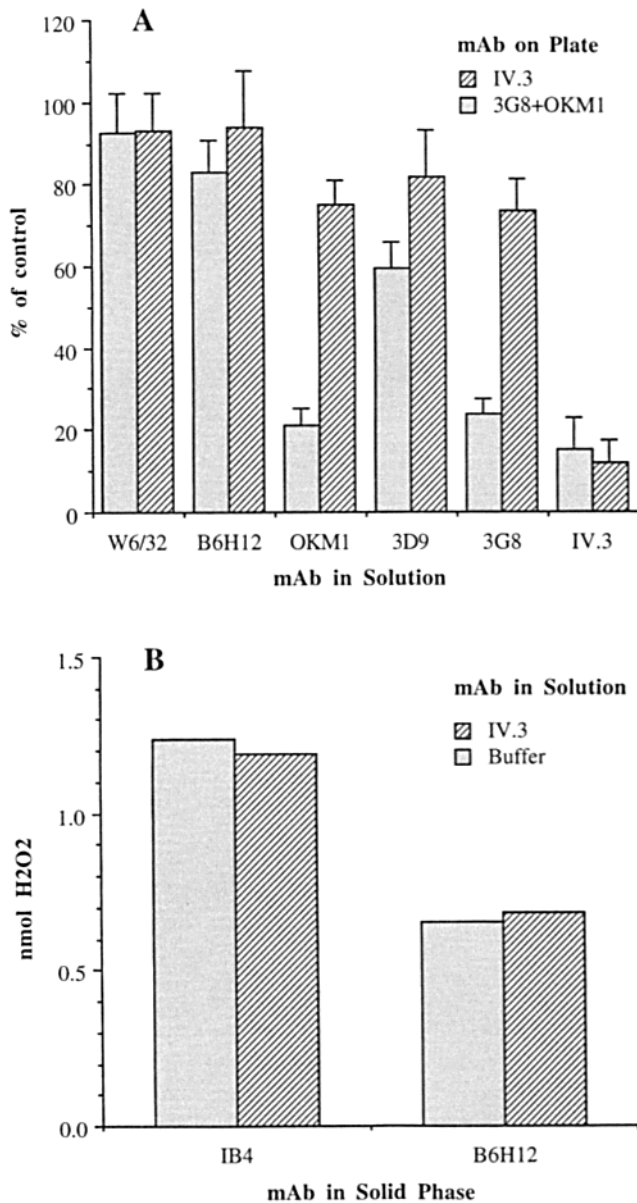
Figure 3. mAb inhibition of the synergistic and the anti-FcγRII-stimulated respiratory burst. (*A*) PMN, pretreated with 5 μg/ml of Fab fragments (W6/32, IV.3, and OKM1), or 2.5 μg/ml of F(ab)₂ fragments (B6H12 and 3G8) or 5 μg/ml intact IgG1 (3D9) at 4°C for 15 min, were incubated in wells coated with anti-FcγRII (*dark bars*) or the combination of anti-CR3 and anti-FcγRIII (*light bars*). The amount of H₂O₂ generated at 60 min is presented as the % of control PMN which were pretreated with buffer containing no mAb (1.33 ± 0.29 nmol in microwells coated with anti-FcγRII and 1.21 ± 0.28 nmol microwells coated with anti-FcγRIII and anti-CR3). Data are means ± SEM of three independent experiments. (*B*) PMN, pretreated with IV.3 Fab, were incubated in wells coated with IB4 (anti-CD18) or B6H12 (anti-IAP), both of which are known to initiate a respiratory burst (4,44). A single experiment, performed in triplicate is shown.

Direct Activation of FcγRII-mediated Respiratory Burst Is Pharmacologically Distinct from the Synergistic CR3-FcγRIII-mediated Respiratory Burst

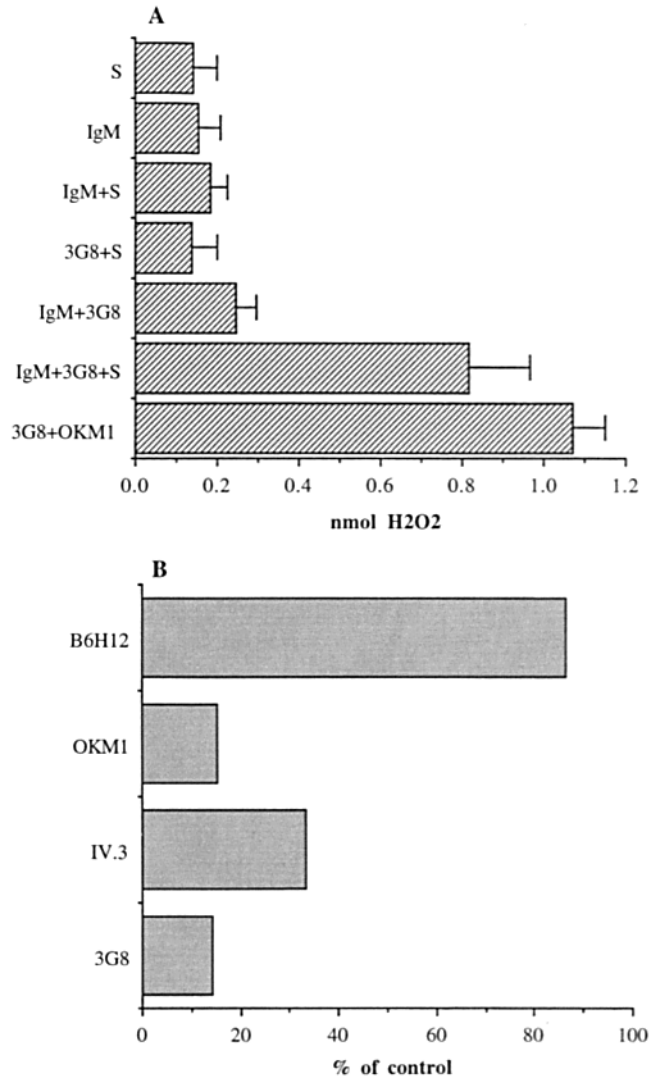To begin to understand the signal transduction pathways in-

Figure 4. iC3b and anti-FcγRIII synergize to stimulate a PMN respiratory burst. (*A*) Adherent surfaces were opsonized with serum alone (*S*); IgM alone (*IgM*); IgM + serum (*IgM +S*); 3G8 anti-FcγRIII + serum (*3G8 + S*); IgM + 3G8; IgM + 3G8 + serum (*IgM+3G+S*); or OKM1 anti-CR3 + 3G8, as described in Materials and Methods. H₂O₂ was assayed 60 min after addition of PMN to the various wells in the presence of a blocking concentration (5 μg/ml) of 3D9 anti-CR1. Wells which were incubated with IgM and serum (±*3G8*) had detectable deposition of C3; the other conditions did not lead to C3 deposition (not shown). Data are mean ± SEM of three independent experiments, each performed in triplicate. In *B*, PMN were pretreated with Fab or F(ab')₂ fragments of various mAb as in Fig. 2, and then assayed for H₂O₂ production after 60 min incubation microwells coated with IgM, serum, and 3G8 as in *A*. A single experiment, performed in triplicate is shown.

volved in the synergistic respiratory burst, we compared the effects of several inhibitors on the synergistic respiratory burst with the burst directly activated by anti-FcγRII. As shown in Fig. 6, NADPH oxidase activation by either adherent stimulus was markedly diminished by both the serine/threonine kinase inhibitor H7 and by the tyrosine kinase inhibitor herbimicin. All known pathways for oxidase activation are inhibited by H7, presumably because assembly of the
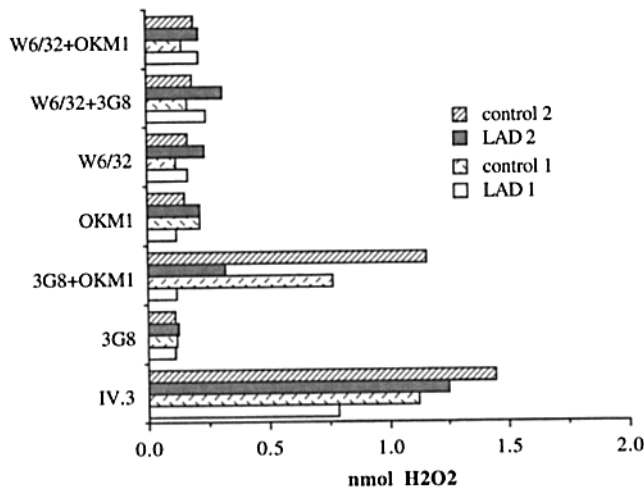
Figure 5. mAb-stimulated respiratory burst in LAD PMN. The generation of H_2O_2 by PMN incubated in microwells coated with various mAbs alone and in combination is compared for normal (*control*) and β_2 deficient (*LAD*) PMN. H_2O_2 generation was assessed after 60 min incubation. A single experiment, in triplicate, was performed on PMN from each of two LAD patients and the normal controls which accompanied them. Anti-FcγRII stimulated a normal respiratory burst in LAD PMN but neither anti-CR3 nor anti-FcγRIII individually, nor their combination, led to a respiratory burst in LAD cells.

Figure 6. Direct activation of FcγRII-mediated respiratory burst is pharmacologically distinct from the synergistic CR3-FcγRIII-mediated respiratory burst. PMN were treated with various pharmacologic inhibitors as described in Materials and Methods and incubated for 60 min at 37°C in mAb-coated wells. Data are presented as the % of control PMN which were incubated with vehicle or buffer. The respiratory burst stimulated by FcγRII and CR3/FcγRIII without drugs was 1.34 ± 0.36 and 1.18 ± 0.26 nmol, respectively, at 60 min. Data are the means $\pm$ SEM of three independent experiments. $2.5\ \mu g/ml$ Cytochalasin D gave identical results to cytochalasin B (data not shown).

oxidase requires protein kinase C-mediated phosphorylation of p47^{phox} (32). However, herbimicin is selective, since the respiratory burst upon LRI/IAP ligation is insensitive to tyrosine kinase inhibitors (44). This suggests that both direct activation of FcγRII and its indirect activation through simultaneous ligation of FcγRIII and CR3 require tyrosine phosphorylation for generation of the respiratory burst. Activation of the respiratory burst by direct ligation of FcγRII was sensitive to pertussis toxin, consistent with the possibility that a trimeric G protein is required for activation. In contrast, the synergistic respiratory burst from ligation of CR3 and FcγRIII was insensitive to pertussis (Fig. 6). Conversely, cytochalasins B and D did not affect the respiratory burst stimulated by direct ligation of FcγRII, but totally abolished the synergistic respiratory burst (Fig. 6). These data suggest that NADPH oxidase activation is dependent on G protein activation when FcγRII is ligated directly and on assembly of the actin cytoskeleton when the FcγRIII/CR3 pathway is activated.

PMN Adhesion via CR3 Causes Colocalization of FcγRII with the Actin Cytoskeleton

To determine how FcγRII became involved in the synergistic respiratory burst, we tested for its presence on the adherent surface after solubilization of PMN with Triton-X100. When the Triton-insoluble cell ghosts were stained with Fab of IV.3, marked fluorescence was detected on cells adherent to surfaces coated with KuFc79 (anti-FcγRII) (Fig. 7 *A*); no fluorescence was seen on plates coated with anti-FcγRIII (Fig. 7 *B*), anti-HLA, anti-CD35, or anti-IAP (data not shown). However, significant fluorescence was observed on plates coated with anti-CR3 alone or in combination with
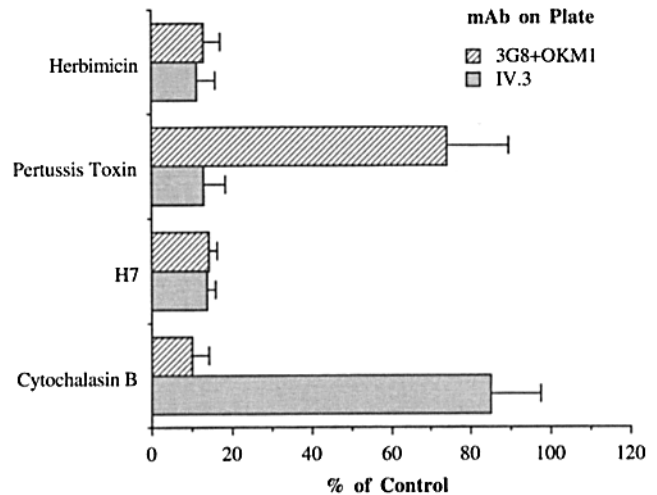
anti-FcγRIII (Fig. 7, *C* and *D*). No fluorescence was seen with W6/32 (anti-HLA) Fab or 2D3 (anti-IAP) F(ab')$_2$ on plates coated with KuFc79 or OKM1 (not shown), demonstrating that not all membrane proteins were insoluble in Triton-X100 in PMN adherent to these surfaces. The assay could be made semi-quantitative by measuring the total fluorescence in each well with a fluorescent plate reader (Fig. 8). When PMN were adherent to anti-FcγRIII/anti-CR3-coated surfaces, significant FcγRII was found associated with the Triton-insoluble cytoskeleton (Fig. 8). This was ~30% of the FcγRII associated with the adherent surfaces of PMN in wells directly coated with anti-FcγRII mAb (Fig. 8). Anti-FcγRIII was neither necessary nor sufficient for this localization of FcγRII to the detergent insoluble cytoskeleton. Anti-CR3 alone or in combination with the irrelevant anti-HLA caused equal retention of FcγRII after Triton solubilization (Fig. 8). No FcγRII above background was found associated with the Triton-insoluble cytoskeletons of PMN adherent to anti-FcγRIII, with or without anti-HLA (Figs. 7 and 8).

To confirm the data from the fluorescence assay, the Triton-insoluble cytoskeletons were radiolabeled with ^{125}I, solubilized, and immunoprecipitated with anti-FcγRII mAb. As shown in Fig. 9, FcγRII could be immunoprecipitated from the Triton-insoluble ghosts of PMN plated onto wells coated with anti-CR3, with or without coligation of FcγRIII or HLA. No FcγRII could be immunoprecipitated from the same preparations of PMN adherent to anti-FcγRIII or anti-HLA-coated surfaces.

These data demonstrated that attachment of PMN to anti-CR3 induces FcγRII to become associated with the adherent membrane in a Triton-resistant manner. To determine
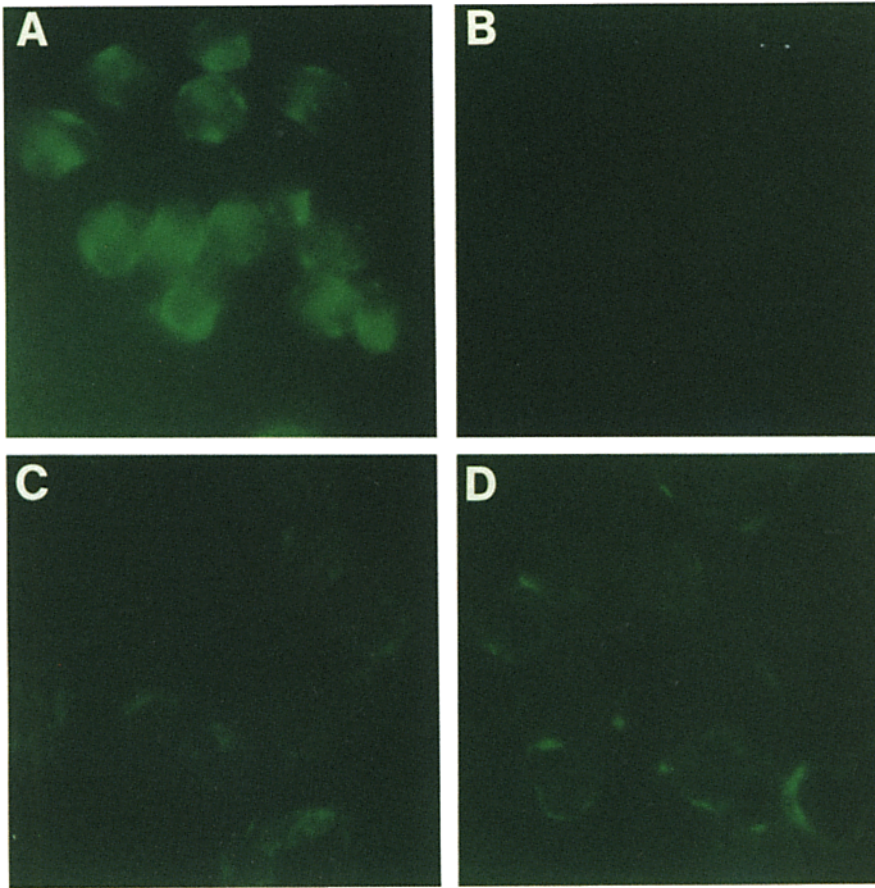
Figure 7. Immunofluorescence stain of FcγRII on mAb-coated slides. PMN were allowed to adhere to wells coated with anti-FcγRII KuFc79 (*A*), anti-FcγRIII 3G8 (*B*), anti-CR3 OKM1 and anti-FcγRIII (*C*), or anti-CR3 OKM1 (*D*) at 37°C for 30 min. After extraction, the Triton-insoluble PMN ghosts were probed with 10 μg/ml of anti-FcγRII IV.3 Fab fragments directly labeled with FITC. A marked FcγRII fluorescence was seen in ghosts from adherent to surfaces coated with KuFc79; no fluorescence was seen on plates coated with anti-FcγRIII (*b*), anti-HLA and anti-IAP (data not shown). FcγRII was found associated with the Triton-insoluble cytoskeleton on plates coated either with anti-CR3 alone or its combination with anti-FcγRIII. Fluorescent Fab of W6/32 did not stain Triton-insoluble ghosts of PMN plated on KuFc79, 3G8, OKM1, or the combination of these antibodies (data not shown).
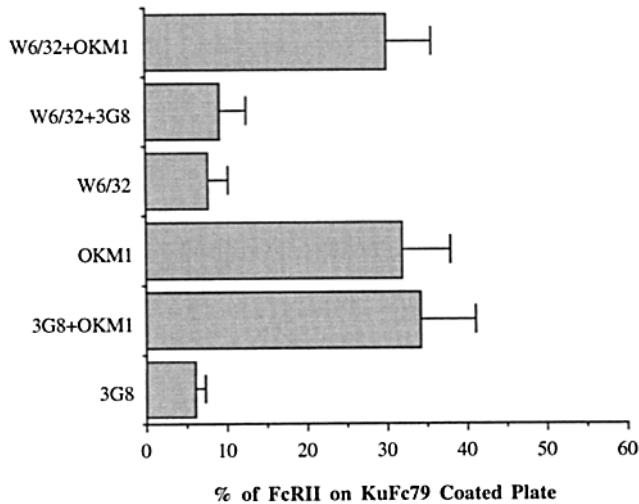

% of FcRII on KuFc79 Coated Plate

Figure 8. Measurements of FcγRII retention with Triton-insoluble cytoskeleton. Adherent PMN ghosts were prepared, extracted, and stained with IV.3 Fab as described in Fig. 7. The total FcγRII fluorescence on each well was determined as described in Materials and Methods. The data for each mAb-coated surface were compared to wells coated with KuFc79. Data shown are the means ± SEM of three independent experiments, each performed in triplicate.

whether this association was affected by inhibitors of the respiratory burst, Triton-insoluble FcγRII was determined in PMN treated with herbimicin, H7, pertussis toxin, and cytochalasins B and D. Herbimicin, pertussis toxin, and H7 had no effect on FcγRII retention in the Triton-insoluble ghosts in PMN adherent to anti-CR3 coated wells (Fig. 10). However, cytochalasin B markedly diminished FcγRII retention (Fig. 10), demonstrating that the actin cytoskeleton is required for stable localization of FcγRII to the adherent membrane of PMN attached to a CR3 ligand. Whether or not anti-FcγRIII also was present on the adherent surface made no difference to the effects of these pharmacologic inhibitors on FcγRII localization. No pharmacologic reagents which inhibited the respiratory burst had any effect on FcγRII retention on anti-FcγRII-coated wells (Fig. 10). Incubation of PMN with IV.3 Fab, which inhibited the respiratory burst (Fig. 3), also inhibited OKM1-dependent localization of FcγRII to the Triton-insoluble cytoskeleton, as assayed by immunoprecipitation with the noncompeting anti- FcγRII mAb KuFc79 (not shown).

Ligation of Both CR3 and FcγRIII Is Required for Tyrosine Phosphorylation of FcγRII

The data suggested that adhesion via CR3 is sufficient to mobilize FcγRII to the actin cytoskeleton, but is not enough
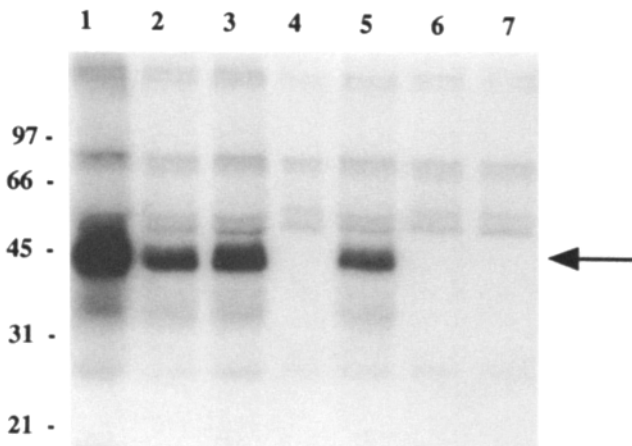
Figure 9. Immunoprecipitation of FcγRII from Triton-insoluble PMN ghosts. Triton-insoluble ghosts of adherent PMN, prepared as described in Materials and Methods, were radiolabeled with ^{125}I, solubilized and immunoprecipitated with anti-FcγRII mAb. Each lane is an immunoprecipitation from PMN ghosts plated onto a different mAb-coated surface. (Lane *1*) Whole cell lysate; (lane 2) anti-CR3 and anti-FcγRIII; (lane 3) anti-CR3 alone; (lane 4) anti-FcγRIII alone; (lane 5) anti-CR3 and anti-HLA; (lane 6) anti-FcγRIII and anti-HLA; (lane 7) anti-HLA alone. FcγRII could be immunoprecipitated from the Triton-insoluble ghosts of PMN onto wells coated with anti-CR3, with or without coligation of FcγRIII or HLA. The arrow indicates intact FcγRII.

to activate an FcγRII-dependent respiratory burst. Since additional adhesion via FcγRIII is also necessary to activate the respiratory burst, and since the synergistic respiratory burst can be inhibited by herbimicin, we tested whether FcγRIII ligation might induce tyrosine phosphorylations required for respiratory burst activation. Since FcγRII is tyrosine-phosphorylated by cross-linking (13, 26), we examined
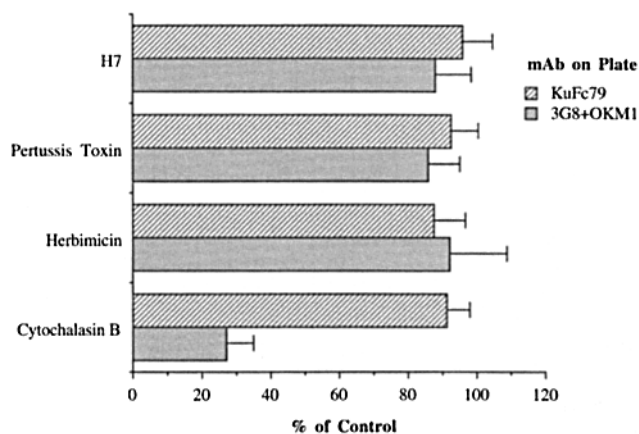

Figure 10. Effects of pharmacologic agents on FcγRII retention in the Triton-insoluble cytoskeleton. PMN, pretreated with various agents as described in Materials and Methods, were processed exactly as in Fig. 7. In each case, the IV.3 Fab fluorescence of Triton-insoluble ghosts from PMN treated with various pharmacologic agents was compared to vehicle- or buffer-treated controls for PMN plated onto KuFc79- and 3G8 + OKM1-coated plates. Data are the means ± SEM of three independent experiments, each performed in triplicate. Cytochalasin D gave identical results to cytochalasin B (data not shown).
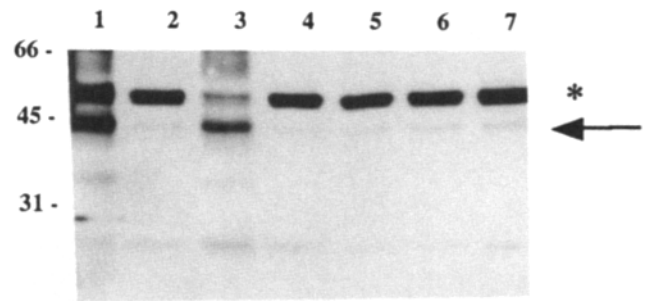

Figure 11. Tyrosine phosphorylation of FcγRII. PMN were adhered to wells coated with immune complexes (lane *1*); anti-FcγRIII (lane 2); anti-CR3 and anti-FcγRIII (lane 3); anti-CR3 (lane 4); anti-HLA (lane 5); anti-HLA and anti-CR3 (lane 6); anti-HLA and anti-FcγRIII (lane 7). After solubilization and immunoprecipitation with anti-FcγRII, phosphotyrosine was detected with anti-phosphotyrosine mAb. The arrow indicates intact FcγRII. The star indicates the heavy chain of IV.3 IgG.

FcγRII tyrosine phosphorylation after PMN adhesion to surfaces coated with anti-FcγRIII, anti-CR3, or the combined ligands. Adherent cells were lysed, immunoprecipitated with anti-FcγRII, and then blotted with anti-phosphotyrosine mAb to detect tyrosine phosphorylation of FcγRII. Tyrosine phosphorylation of FcγRII was enhanced only when both ligands were present or when PMN were exposed to immune complexes (Fig. 11).

Discussion

IgG antibody and complement component 3 (C3) are the two major opsonins in human serum. Although collaboration between receptors for antibody and complement is likely to have an important role in host defense, and synergy between antibody and complement in activating effector functions of phagocytes has been known for 20 years, the molecular mechanisms involved in this interaction are completely unknown. In the last few years, a molecular description of the various receptors for IgG Fc and C3 has been completed. This detailed understanding of the receptors has allowed a finer analysis of the effector functions triggered by these receptors. In this work, we have examined the contribution of CR3 and FcγRIII to assembly of the NADPH oxidase. Previous work has shown that ligation of FcγRII, but not FcγRIII or CR3 leads to activation of the respiratory burst (4, 27, 28). Surprisingly, we found that when mAb specific for FcγRIII and CR3 were both used to coat a surface, significant H_2O_2 generation occurred, comparable to the amount produced when PMN were stimulated by anti-FcγRII. Activation of the respiratory burst was specific for this combination of antibodies, since neither anti-HLA nor anti-CR1 could substitute for either anti-FcγRIII or anti-CR3; activation also required both mAb to be on the tissue culture dish. Strangely, the respiratory burst generated by synergy between FcγRIII and CR3 ligation was completely inhibited by Fab of anti-FcγRII. This led us to consider the possibility that the synergistic effect was an artifact of engagement of FcγRII by free Fc fragments of the antibodies used to coat the activating surfaces. However, the evidence against this artifact is very strong: (*a*) only this combination of antibodies works, since a variety of isotype controls and

mAb which bind PMN alone and in combination could not activate the respiratory burst; (b) coating plates with either of the antibodies alone could not activate the respiratory burst at any dose; (c) the dose response curves for the synergistic effect showed inhibition at high doses for both antibodies, which is a circumstance in which exposed Fc fragments, if significant, should be increased; and (d) LAD cells, which were perfectly competent to mount a respiratory burst when FcγRII was engaged, were incapable of activating the respiratory burst when adherent to wells coated with anti-FcγRIII and anti-CR3. Moreover, anti-FcγRIII could synergize with deposited iC3b as well as anti-CR3 for assembly of the NADPH oxidase. Finally, direct engagement of FcγRII activated the respiratory burst by a pathway that was pharmacologically distinguishable from the synergistic respiratory burst. While H_2O_2 production in response to direct FcγRII ligation was inhibited by pertussis toxin, the synergistic respiratory burst was not; conversely, synergistic activation of the NADPH oxidase was inhibited by cytochalasin B, while activation in response to direct FcγRII ligation was not. Thus, these data demonstrated that the synergistic respiratory burst requires engagement of both FcγRIII and CR3 and uses a pathway which involves FcγRII but is distinct from the signaling cascade engaged by direct ligation of FcγRII.

Inhibition of the synergistic respiratory burst by cytochalasin B and herbimicin suggested that, in addition to FcγRII, both the actin cytoskeleton and tyrosine phosphorylation were important in signal transduction for this pathway of activating the NADPH oxidase. We examined colocalization of FcγRII with the Triton-X100-insoluble cytoskeleton in PMN adherent to surfaces coated with either anti-FcγRIII or anti-CR3. These experiments demonstrated that some FcγRII became Triton-X100-insoluble when PMN were adherent to anti-CR3-coated surfaces, and the presence of cytoskeleton-associated FcγRII was unaffected by whether or not anti-FcγRIII was also present. Since FcγRII colocalization with CR3 was abolished by cytochalasin B, it is more likely that these receptors are indirectly associated, through linkage to the cytoskeleton, than that a stable direct intermolecular interaction between FcγRII and CR3 exists, as has been proposed for CR3 and FcγRIII (43). Finally, since FcγRII association with the actin cytoskeleton was signaled by adhesion via CR3 but H_2O_2 production was not, we hypothesized that cytoskeletal association of FcγRII was necessary but not sufficient for an FcγRII-dependent respiratory burst.

FcγRIII on PMN is a phosphoinositol glycan (PIG)-linked, and not a transmembrane, protein (39). Recently, experimental data from several groups have suggested an association of PIG-linked proteins with particular membrane domains rich in tyrosine kinases (8, 37). Indeed, crosslinking of PIG-linked proteins can lead to tyrosine phosphorylation and cell proliferation (7). Since it is known that FcγRII can itself become phosphorylated upon ligation, we reasoned that the role for FcγRIII in the synergistic respiratory burst might be to initiate FcγRII functions by inducing tyrosine phosphorylation on the adherent cell surface. Indeed, tyrosine phosphorylation of CR3-immobilized FcγRII occurred only when FcγRIII also was ligated on the basal PMN surface. Although FcγRII is not necessarily the tyrosine kinase target required for oxidase assembly, there is no evidence against this possibility. Certainly, the proximity between FcγRII and a tyrosine kinase is required for initiation of NADPH oxidase assembly by either FcγRIII/CR3 synergy or direct activation of FcγRII. Thus, we suggest that the mechanism by which FcγRIII and CR3 cooperate in generation of the PMN respiratory burst is that CR3 ligation induces cytoskeletal association of FcγRII, and FcγRIII is required for a critical tyrosine phosphorylation, of FcγRII itself or of some substrate closely associated with FcγRII.

When the synergistic respiratory burst is activated, the association of FcγRII with cytoskeleton is correlated with signaling for NADPH oxidase assembly. In contrast, when FcγRII is ligated directly, there is an alternative, pertussis toxin inhibitable, pathway for tyrosine kinase activation, not inhibitable by cytochalasin B. Whether the tyrosine kinase(s) involved in the two pathways are identical is unknown. The association of tyrosine kinases with PIG-linked proteins is likely to be independent of the exact nature of the extracellular domain, since the PIG anchors are quite similar among this protein family (15, 18). However, antibody to decay accelerating factor, another PIG-linked protein on PMN, cannot substitute for anti-FcγRIII in the synergistic respiratory burst (unpublished data). This may imply that there are additional requirements for activation of the respiratory burst, such as interaction between the extracellular domains of FcγRIII and CR3 (43). In addition, FcγRIII ligation can initiate signal transduction and affect FcγRII function, presumably without the participation of CR3 (6, 29, 36). There are about 10-fold more FcγRIII than FcγRII expressed on PMN (27). Thus, a pathway of PMN activation involving coligation of FcγRIII and CR3 is likely to be critically important in circumstances in which IgG opsonization is limiting. This would be most likely early in the immune response, when the class switch from IgM to IgG is just beginning and when IgG affinity is low, but complement activation is efficient. However, it is notable that the PMN respiratory burst induced by binding to adherent immune complexes is pertussis-insensitive (5, 14), suggesting that the synergistic pathway for oxidase activation may be of even more general significance. This synergistic pathway for respiratory burst activation also may be critical for PMN tumor cytotoxicity for which CR3, FcγRII, and FcγRIII are all required (30).

References

1. Aderem, A. A., S. D. Wright, S. C. Silverstein, and Z. A. Cohn. 1985. Ligated complement receptors do not activate the arachidonic acid cascade in resident peritoneal macrophages. J. Exp. Med. 161:617–622.
2. Barnstable, C. J., W. F. Bodmer, G. Brown, et al. 1978. Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens- new tools for genetic analysis. Cell. 14:9–17.
3. Berger, M., J. J. O'Shea, A. S. Cross, et al. 1984. Human neutrophils increase expression of C3bi as well as C3b receptors upon activation. J. Clin. Invest. 74:1566–1571.
4. Berton, G., C. Laudanna, C. Sorio, and F. Rossi. 1992. Generation of signals activating neutrophil functions by leukocyte integrins: LFA-1 and

gp150/95, but not CR3, are able to stimulate the respiratory burst of human neutrophils. *J. Cell Biol.* 116:1007–1017.

5. Blackburn, W. D., and L. W. Heck. 1989. Neutrophil activation by surface bound IgG is via a pertussis toxin insensitive G protein. *Biochem. Biophys. Res. Commun.* 164:983–989.

6. Boros, P., J. A. Odin, T. Muryoi, S. K. Masur, C. Bona, and J. C. Unkeless. 1991. IgM anti-FcgammaR autoantibodies trigger neutrophil degranulation. *J. Exp. Med.* 173:1473–1482.

7. Brown, D. 1993. The tyrosine kinase connection: how GPI-anchored proteins activate T cells. *Curr. Opin. Immunol.* 5:349–354.

8. Brown, D. A., and J. K. Rose. 1992. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell.* 68:533–544.

9. Brown, E. J. 1989. Binding and internalization of C3b oligomers by human polymorphonuclear leukocytes. *J. Biol. Chem.* 264:6196–6201.

10. Brown, E. J., J. F. Bohnsack, and H. D. Gresham. 1988. Mechanism of inhibition of immunoglobulin G-mediated phagocytosis by monoclonal antibodies that recognize the Mac-1 antigen. *J. Clin. Invest.* 81:365–375.

11. Brown, E. J., L. Hooper, T. Ho, and H. D. Gresham. 1990. Integrin-associated protein: a 50-kD plasma membrane antigen physically and functionally associated with integrins. *J. Cell Biol.* 111:2785–2794.

12. Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteiolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* 252:1102–1106.

13. Connelly, P. A., C. A. Farrell, J. M. Merenda, M. J. Conklyn, and H. J. Showell. 1991. Tyrosine phosphorylation is an early signaling event common to Fc receptor crosslinking in human neutrophils and rat basophilic leukemia cells (RBL-2H3). *Biochem. Biophys. Res. Commun.* 177:192–201.

14. Crockett-Torabi, E., and J. C. Fantone. 1990. Soluble and insoluble immune complexes activate human neutrophil NADPH oxidase by distinct Fcgamma receptor-specific mechanisms. *J. Immunol.* 145:3026–3032.

15. Cross, G. A. M. 1990. Glycolipid anchoring of plasma membrane proteins. *Annu. Rev. Cell Biol.* 6:1–39.

16. De la Harpe, J., and C. F. Nathan. 1985. A semi-automated micro-assay for H2O2 release by human blood monocytes and mouse peritoneal macrophages. *J. Immunol. Methods.* 78:323–336.

17. Ehlenberger, A. G., and V. Nussenzweig. 1977. The role of membrane receptors for C3b and C3d in phagocytosis. *J. Exp. Med.* 145:357–371.

18. Ferguson, M. A. J. 1988. Cell-surface anchoring of proteins via glycosyl-phosphatidylinositol structures. *Annu. Rev. Biochem.* 57:285–320.

19. Fleit, H. B. 1991. Monoclonal antibodies to human neutrophil FcgammaRIII (CD16) identify polypeptide epitopes. *Clin. Immunol. Immunopathol.* 59:222–235.

20. Fleit, H. B., S. D. Wright, and J. C. Unkeless. 1982. Human neutrophil Fc gamma receptor distribution and structure. *Proc. Natl. Acad. Sci. USA.* 79:3275–3279.

21. Graham, I. L., and E. J. Brown. 1991. Extracellular calcium results in a conformational change in Mac-1 (CD11b/CD18) on neutrophils: differentiation of adhesion and phagocytosis functions of Mac-1. *J. Immunol.* 146:685–691.

22. Graham, I. L., H. D. Gresham, and E. J. Brown. 1989. An immobile subset of plasma membrane CD11b/CD18 (Mac-1) is involved in phagocytosis of targets recognized by multiple receptors. *J. Immunol.* 142:2352–2358.

23. Greenberg, S., and S. C. Silverstein. 1993. Phagocytosis. *In* Fundamental Immunology. W. E. Paul, editor. Raven Press Ltd., New York. p. 941–964.

24. Gresham, H. D., J. L. Goodwin, D. C. Anderson, and E. J. Brown. 1989. A novel member of the integrin receptor family mediates Arg-Gly-Asp-stimulated neutrophil phagocytosis. *J. Cell Biol.* 108:1935–1943.

25. Gresham, H. D., I. L. Graham, D. C. Anderson, and E. J. Brown. 1991. Leukocyte adhesion deficient (LAD) neutrophils fail to amplify phagocytic function in response to stimulation: evidence for CD11b/CD18-dependent and -independent mechanisms of phagocytosis. *J. Clin. Invest.* 88:588–597.

26. Huang, M.-M., Z. Indik, L. F. Brass, J. A. Hoxie, A. D. Schreiber, and J. S. Brugge. 1992. Activation of FcγRII induces tyrosine phosphoryla-

tion of multiple proteins including Fc[gamma]RII. *J. Biol. Chem.* 267:5467–5473.

27. Huizinga, T. W., K. M. Dolman, N. J. van der Linden, et al. 1990. Phosphatidylinositol-linked FcRIII mediates exocytosis of neutrophil granule proteins, but does not mediate initiation of the respiratory burst. *J. Immunol.* 144:1432–1437.

28. Huizinga, T. W., F. van Kemenade, L. Koenderman, et al. 1989. The 40-kDa Fc gamma receptor (FcRII) on human neutrophils is essential for the IgG-induced respiratory burst and IgG-induced phagocytosis. *J. Immunol.* 142:2365–2369.

29. Kimberly, R. P., J. W. Ahlstrom, M. E. Click, and J. C. Edberg. 1990. The glycosyl phosphatidylinositol-linked Fc gamma RIIIon PMN mediates transmembrane signaling events distinct from Fc gamma RII. *J. Exp. Med.* 171:1239–1255.

30. Kushner, B. H., and N.-K. V. Cheung. 1992. Absolute requirement of CD11/CD18 adhesion molecules, FcRII, and the phosphatidylinositol-linked FcRIII for monoclonal antibody-mediated neutrophil antihuman tumor cytotoxicity. *Blood.* 79:1484–1490.

31. Looney, R. J., D. H. Ryan, K. Takahashi, et al. 1986. Identification of a second class of IgG Fc receptors on human neutrophils. A 40 kilodalton molecule also found on eosinophils. *J. Exp. Med.* 163:826–836.

32. Nauseef, W. M., B. D. Volpp, S. McCormick, K. G. Leidal, and R. A. Clark. 1991. Assembly of the neutrophil respiratory burst oxidase. Protein kinase C promotes cytoskeletal and membrane association of cytosolic oxidase components. *J. Biol. Chem.* 266:5911–5917.

33. O'Shea, J. J., E. J. Brown, B. E. Seligmann, J. A. Metcalf, M. M. Frank, and J. I. Gallin. 1985. Evidence for distinct intracellular pools of receptors for C3b and C3bi in human neutrophils. *J. Immunol.* 134:2580–2587.

34. Parham, P. 1983. On the fragmentation of monoclonal IgG1, IgG2a, and IgG2b from Balb/c mice. *J. Immunol.* 131:2895–2902.

35. Rosales, C., and E. J. Brown. 1991. Two mechanisms for IgG Fc-receptor-mediated phagocytosis by human neutrophils. *J. Immunol.* 146:3937–3944.

36. Salmon, J. E., N. L. Brogle, J. C. Edberg, and R. P. Kimberly. 1991. Fcgamma receptor III induces actin polymerization in human neutrophils and primes phagocytosis mediated by Fcgamma receptor II. *J. Immunol.* 146:997–1004.

37. Sargiacomo, M., M. Sudol, Z. Tang, and M. P. Lisanti. 1993. Signal transducing molecules and glycosyl-phosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells. *J. Cell Biol.* 122:789–807.

38. Sehgal, G., K. Zhang, R. F. Todd, L. A. Boxer, and H. R. Petty. 1993. Lectin-like inhibition of immune-complex receptor-mediated stimulation of neutrophils—effects on cytosolic calcium release and superoxide production. *J. Immunol.* 150:4571–4580.

39. Selvaraj, P., W. F. Rosse, R. Silber, and T. A. Springer. 1988. The major Fc receptor in blood has a phosphatidylinositol anchor and is deficient in paroxysmal nocturnal haemoglobinuria. *Nature (Lond.).* 333:565–567.

40. Willis, H. E., B. Browder, A. J. Feister, T. Mohanakumar, and S. Ruddy. 1988. Monoclonal antibody to human IgG Fc receptors. Cross-linking of receptors induces lysosomal enzyme release and superoxide generation by neutrophils. *J. Immunol.* 140:234–239.

41. Wright, S. D., and S. C. Silverstein. 1983. Receptors for C3b and C3bi promote phagocytosis but not the release of toxic oxygen from human phagocytes. *J. Exp. Med.* 158:2016–2023.

42. Wright, S. D., P. E. Rao, C. Wesley, et al. 1983. Identification of the C3bi receptor of human monocytes and macrophages by using monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 80:5699–5703.

43. Zhou, M.-J., and E. J. Brown. 1993. Leukocyte response integrin and integrin associated protein act as a signal transduction unit in generation of a phagocyte respiratory burst. *J. Exp. Med.* 178:1165–1174.

44. Zhou, M., R. F. Todd, III, J. G. J. Van de Winkel, and H. R. Petty. 1993. Cocapping of the leukoadhesin molecules complement receptor type 3 and lymphocyte function-associated antigen-1 with Fc$_{gamma}$ receptor III on human neutrophils: possible role of lectin-like interactions. *J. Immunol.* 150:3030–3041.