Cross-linking of the High Affinity Fc Receptor for Human Immunoglobulin G1 Triggers Transient Activation of NADPH Oxidase Activity

CONTINUOUS OXIDASE ACTIVATION REQUIRES CONTINUOUS DE NOVO RECEPTOR CROSS-LINKING*

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Cross-linking of the high affinity Fc receptor for human immunoglobulin G1 (FcyRI) on U937 cells triggered superoxide anion (O2-) release. This was accomplished by the binding of an FcyRI-specific monoclonal antibody, mAb 32, followed by cross-linking of the mAb on the cell with anti-mouse IgG F(ab’)2 by FcyRI-specific mAbs 32 and 22 used as an equimolar mixture or by FcyRI-specific mAb 197 (a murine IgG2a and thus a multivalent ligand for FcyRI) alone. At subsaturating concentrations of the FcyRI-cross-linking ligands, O2- generation was continuous over relatively long intervals. However, saturating concentrations triggered an often substantial but always transient O2- burst. This transient burst of oxidase activity ceased with maximal ligand accumulation on the cell. Cells in which oxidase activity had ceased could be restimulated using phorbol 12-myristate 13-acetate or aggregated human IgG1, indicating that cessation of O2- generation was not due to a generalized exhaustion or inhibition of the NADPH oxidase pathway. Cells incubated in subsaturating concentrations of cross-linking antibodies continued to release O2- until binding of the ligand ceased. In addition, the rates of O2- production and ligand accumulation were the same. Thus, continuous O2- production appeared to be dependent upon continuous de novo formation of cross-linked and activated FcyRI. Furthermore, the mol of O2- released in response to FcyRI cross-linking by the multivalent ligand mAb 197 were directly proportional to the mol of mAb bound over a range of saturating and subsaturating concentrations. This evidence suggests a quantal relationship between each FcyRI activated (cross-linked) and the resultant oxidase activity and supports a "rate" model for the activation of this response. Thus, each FcyRI entering the pool of activated receptors probably makes a unitary contribution to the signal. An additional finding showed that cross-linked FcyRI became associated with the cell cytoskeleton and that this association was also transient. Dissociation of FcyRI from its cytoskeletal attachment occurred well after cessation of O2- production.

The high affinity Fc receptor for human IgG1 (FcyRI)1 is found on monocytes, macrophages, and to a lesser extent, on interferon γ-treated neutrophils (1, 2). This receptor has been shown to mediate some of the responses characteristic of FcyR-stimulated phagocytes, including superoxide anion (O2-) production by monocytes and by differentiated parent (3) and mutant cells2 of the myelomonocytic cell line, U937. However, because the receptors have to be aggregated by multivalent ligands in order to trigger any of the FcyRI-mediated responses, it has been difficult to assess the role of individual receptors in FcyRI activation of the NADPH oxidase. In addition, it has been difficult to determine whether the rates of oxidase activity triggered through FcyRI were a result of continuous receptor signaling by ligand-occupied receptors or whether the rates were due to a transient activation of the oxidase which continued by virtue of continued de novo formation of receptor clusters. As part of our investigation into the relationship between the FcyRI activation and the activation of NADPH oxidase activity, we have used high affinity monoclonal antibodies (mAbs) specific for FcyRI, sometimes coupled with the use of second, anti-mouse IgG F(ab’)2, to cross-link the pool of FcyRI and stimulate O2- generation. Three FcyRI-specific mAbs were used. mAbs 32 (3) and 223 are of the mouse subclass IgG1 and react with FcyRI at different epitopes and at epitopes distinct from the ligand-binding site of this receptor. mAb 197 is a mouse IgG2a antibody that binds with high affinity through its Fc region to the Fc-binding site of FcyRI (4) and which binds through its Fab region to an epitope outside of the Fc-binding site on FcyRI.3 Based on the binding characteristics of these antibodies, it seemed likely that a mixture of mAbs 32 and 22, or mAb 197 alone, would cross-link FcyRI into lattices and induce receptor-mediated functions. We have measured the kinetics of the binding of fluorescein-conjugated anti-FcyRI-specific monoclonal antibodies and the kinetics of the resultant oxidase response. Here, we report on the transience of FcyRI-activated oxidase activity and on the requirement for continuous receptor cross-linking to sustain oxidase activity.

1 The abbreviations used are: FcyRI, the high affinity Fc receptor(s) for human IgG1; mAb(s), monoclonal antibody(ies); hIgG1, human IgG, subclass 1; gom, goat anti-mouse; O2-, superoxide anion; Hapes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FITC, fluorescein isothiocyanate; F-mAb, FITC-conjugated mAb; MFI, mean fluorescence intensity; fMLF, formylmethionylleucylphenylalanine.

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The following cross-linking antibodies were labeled with FITC: mAb 32, mAb F-32; mAb 197; and F(ab') 2 of goat (Fg- 
gm) or rabbit anti-mouse Ab. FITC-labeled mAb 32 was mixed with 
unconjugated mAb 22 for all binding studies using F-32/22 mixtures 
or was used as the precoating antibody in some experiments in which 
crosslinked F-32-FcγRI complexes were cross-linked using unlabelled 
gam F(ab') 2.

Measurement of Cytoskeleton-associated FcγRI

FcγRI were saturated with mAb F-32 at 4 °C. Cells were washed 
three times with gam F(ab') 2 in O 2 assay medium. To determine 
the extent of cytосkeletal attachment, samples of cells were washed 
with phosphate-buffered saline containing 1 mg/ml bovine serum 
albumin, then with phosphate-buffered saline. Volumes of 50 μl 
containing 2 x 10 6 cells were added to 1 ml of extraction buffer: 10 
mm Tris, pH 7.4, 150 mm NaCl, 0.5% Nonidet P-40, 0.1% purified 
bovine serum albumin for 5 min at 4 °C (8). Detergent-insoluble 
material (cytoskeletons) was sedimented at 150 × g for 5 min, washed 
twice with extraction buffer, and fixed with 1% paraformaldehyde 
in a volume of 0.2 ml. Cytoskeletons were analyzed by flow cytometry 
as described above. Cytoskeleton-associated FcγRI is expressed as 
the mean fluorescence intensity of 2000 cytoskeletons per data point 
after subtraction of autofluorescence.

RESULTS

Kinetics of O 2 Generation Stimulated by Cross-linking 32-
FcγRI Complexes with Various Concentrations of Anti-mouse 
IgG Antibody—Since a functional consequence of receptor 
cross-linking is the activation of the NADPH oxidase, O 2 
production could be used as a functional measurement of receptor 
cross-linking. To establish the optimal effective concentra-
tion of cross-linking antibody with respect to the activa-
tion of the NADPH oxidase, FcγRI on differentiated 
A12.13 cells were exposed to a saturating concentration of an 
FcγRI-specific monoclonal antibody, mAb 32. FcγRI-bound 
mAb 32 were cross-linked by various concentrations of gam 
F(ab') 2. Fig. 1A shows the time course of O 2 generation that 
was triggered by different amounts of second antibody. O 2 
production at 3, 9, and 40 min is shown in Fig. 1B as a 
function of the concentration of gam F(ab') 2. mAb 32 alone 
did not trigger O 2 release. These results indicate that there 
were two second antibody concentration-dependent phenom-
ena: (a) a linear and concentration-dependent release of O 2 
over 90 min triggered by concentrations of second antibody 
in the range of 0.1-1 μg/ml with an "optimal" effective concen-
tration at approximately 0.2 μg/ml; and (b) a burst of O 2 
released within the first 3 min of stimulation by concentrations 
higher than 2 μg/ml at an "optimal" effective concentration of 
approximately 20 μg/ml. Furthermore, O 2 production 
(Fig. 1B) was dependent on the concentration of cross-
linking antibody prior to cessation of O 2 production. High 
levels of cytochrome c reduction at the later time points 
indicate that cessation of O 2 generation was not due to a 
depletion of oxidized cytochrome c.

To determine whether the second antibody used to cross-
link mAb 32 on cells saturated all of the binding sites on mAb 
32 during the brief burst of oxidative activity, mAb 32-FcγRI 
complexes were cross-linked with a high concentration of gam 
F(ab') 2. This cross-linking produced an oxidative response 
(Fig. 2A) and at the same time rapidly saturated epitopes on the 
receptor-bound mAb 32 (Fig. 2B). Therefore, the oxidative 
burst triggered by the high concentration of second an-
tibody coincided with saturation of FcγRI-bound mAb 32.

Kinetics of O 2 Generation Stimulated by Various Concentrations 
of FcγRI-specific mAbs—To determine whether the bi-
modal response to second antibody cross-linking (Fig. 1B) 
was characteristic of the normal cell response to cross-linking 
of FcγRI, the time course of O 2 generation triggered by 
alternate FcγRI-cross-linking antibodies was measured. Ear-

EXPERIMENTAL PROCEDURES

Cell Preparation

A12.13 cells, high expression mutants of U937 cells for FcγRI and 
O 2, 7 were incubated with 100 units/ml recombinant human interferon 
γ (a gift from Genentech, San Francisco) in RPMI 1640 supplemented 
with 10% heat-inactivated fetal calf serum (GIBCO) for 4-5 days 
before use.

mAb Preparation

All mAbs were purified from ascites by high pressure liquid chro-
matographic separation on a DEAE-5PW column and by chro-
matographic separation on a protein A-Sepharose column.

Methods for Cross-linking FcγRI by the Use of 
Cross-linking Antibodies

Indirect Cross-linking—Cells were incubated with 10 μg/ml mAb 
32 in RPMI/fetal calf serum containing 20 mM Hepes at 4 °C for 60 
min. mAb 32-coated cells were washed three times. FcγRI-mAb 32 
complexes on the coated cells were cross-linked with various concen-
trations of goat anti-mouse IgG F(ab') 2 (gam F(ab') 2 ) (Cappel, West 
Chester, PA), as indicated in the text.

Direct Cross-linking, Method 1—Cells were incubated with various 
concentrations of an equimolar mixture of FcγRI-specific mAbs 32 and 
22. mAbs 32 and 22, both mouse antibodies of the IgGl isotype, 
bind two different epitopes on FcγRI through the mAb Fab regions. 3

Direct Cross-linking, Method 2—Cells were incubated with various 
concentrations of FcγRI-specific mAb 191, a murine IgG2a, which 
binds an epitope on FcγRI through its Fab regions and, with high 
affinity, the Fc-binding site of FcγRI through its Fc-binding domain. 5

Microscopic observation indicated that incubation of cells with mAbs 
32 and 22 or mAb 197 did not cause cell aggregation.

Superoxide Anion Assay

The method that was used to measure O 2 is a modification of the 
method of Babiory et al. (5). Cells were washed and preincubated in 
O 2 assay medium (20 mM Hepes buffered with 10 mM KOH, 137 mM 
NaCl, 1 mM MgCl 2, 1 mM CaCl 2, pH 7.4) (HBS), containing 5.5 mM 
glucose and 200 mM KCN (6). Cell suspensions were mixed with 
ferricytochrome c (cytochrome c: type III, Sigma) and stimulant 
(FcγRI-cross-linking antibodies or other stimulatory agents) or me-
dium to final concentrations: cytochrome c, 0.5 mg/ml; cells, 10 6/ml; 
and stimulant, as indicated in the text, all in a volume of 1-1.5 ml. 
Samples of 150-200 μl were taken at various intervals into a 96-well 
plate. Reduced cyt observing c in the samples was measured by absorp-
tion difference at 550 nm using a Dynatech MR 700 well-plate reader 
set in a dual wavelength mode. Values obtained at the reference 
wavelength (630 nm) and from control incubations lacking stimulant 
were subtracted. An extinction coefficient of A 550 = 1.46 x 10 5 M -1 
cm -1, established previously for the 10-nm bandwidth of the Dynatech 
550-nm filter (Footnote 2; method in Ref. 7), was used to quantitate 
reduced cyt observing c. In an alternative procedure, assay volumes of 
6 ml were used, and 1-ml samples were centrifuged at various times. 
Reduced cyt observing c in 300-μl volumes of cell-free supernatant was 
measured with a well-plate reader, as above. In some experiments, 20 
mM NH 4 Cl was substituted for 20 mM NaCl in the O 2 assay medium, 
as indicated in this section in order to inhibit lysosomal degradation 
of internalized cross-linking antibody. Evidence for the inhibition of 
reoxidation of reduced cyt observing c by KCN, an inhibitor of mito-
ochondrial cyt observing c oxidase, and the quantitation of reduction 
is described under "Results."

Measurement of Cell-associated FcγRI-cross-linking Antibody

Cells were stimulated to generate O 2 by directly or indirectly cross-
linking FcγRI with cross-linking antibody. To measure the amount 
of cross-linking antibody associated with the cells, FITC-conjugated 
was detected through a 530-nm long pass filter, and the mean fluo-
rescence intensity (MFI) was computed from data expressed on a 
linear fluorescence scale of 0-1000. Autofluorescence was subtracted.
duplicate samples did not exceed expressed as the mean fluorescence intensity. Variation between exposed at of 1 mg/ml hIgG1 precoated cells were incubated in an F(ab'), had been subtracted from the some cells were bound by mAb 32 as in Fig. 1. Control and mAb 32-fluorescence associated with unfixed cells was measured by cytofluorimetry using various concentrations of gam F(ab'),. Additional control and mAb32-precoated cells were incubated in F(ab'), gam. Additional control and mAb32-precoated cells were incubated in F(ab'), at 37 °C for 2 min (Cyt) c rapidly saturates FcyRI-bound mAb 32. The cells were centrifuged, and the reduced cytochrome c was measured at the indicated times. The zero time point was omitted from the graph. Listed units are pg of gam F(ab')*/ml of assay medium.

Transient activation of NADPH oxidase activity

Evidence that a high stimulatory concentration of gam F(ab')2 rapidly saturates FcyRI-bound mAb 32. FcyRI on some cells were bound by mAb 32 as in Fig. 1. Control and mAb 32-precoated cells were incubated in an O2 production assay ±10 μg/ml gam F(ab'). The cells were centrifuged, and the reduced cytochrome (Cyt) c in the supernatant was measured (A). The cell pellets were exposed at 4 °C for 60 min to F-rabbit am (FITC-am) in the presence of 1 mg/ml hlgG1 (B) to detect bound mAb 32 unblocked by the F(ab')2 gam. Additional control and mAb32-precoated cells were incubated in O2 generation assay medium with 10 μg/ml FITC-gam F(ab')2 at 37 °C for 2 min (C, and 3rd bars) or 37 °C for 15 min (C, 2nd and 4th bars). These cells were washed immediately after the incubation, and the O2 generation assay medium was discarded. Fluorescence associated with unfixed cells was measured by cytofluorography, as described under "Experimental Procedures," and is expressed as the mean fluorescence intensity. Variation between duplicate samples did not exceed 5% in this assay.

mAb 197 is a third antireceptor antibody. As a member of

FIG. 1. O2 production stimulated by increasing concentrations of polyclonal second antibody. A12.13 cells were exposed to 10 μg/ml mAb 32 at 4 °C for 60 min, then washed. FcyRI mAb 32 complexes were cross-linked at 37 °C in the standard O2 assay containing various concentrations of gam F(ab')2. (A) O2 production (nmol of cytochrome (Cyt) c reduced) was measured at the indicated times. The zero time point was omitted from the graph. Listed units are μg of gam F(ab')2/ml of assay medium. (B) cumulative O2 production as a function of gam F(ab')2 concentration by 3 (○), 9 (□), and 40 (△) min. Cytochrome c reduced in the absence of gam F(ab')2 had been subtracted from the curves in B.

FIG. 2. Evidence that a high stimulatory concentration of gam F(ab')2 rapidly saturates FcyRI-bound mAb 32. FcyRI on some cells were bound by mAb 32 as in Fig. 1. Control and mAb 32-precoated cells were incubated in an O2 production assay ±10 μg/ml gam F(ab'). The cells were centrifuged, and the reduced cytochrome (Cyt) c in the supernatant was measured (A). The cell pellets were exposed at 4 °C for 60 min to F-rabbit am (FITC-am) in the presence of 1 mg/ml hlgG1 (B) to detect bound mAb 32 unblocked by the F(ab')2 gam. Additional control and mAb32-precoated cells were incubated in O2 generation assay medium with 10 μg/ml FITC-gam F(ab')2 at 37 °C for 2 min (C, 1st and 3rd bars) or 37 °C for 15 min (C, 2nd and 4th bars). These cells were washed immediately after the incubation, and the O2 generation assay medium was discarded. Fluorescence associated with unfixed cells was measured by cytofluorography, as described under “Experimental Procedures,” and is expressed as the mean fluorescence intensity. Variation between duplicate samples did not exceed 5% in this assay.

FIG. 3. O2 production triggered by FcyRI-specific mAbs. (A) cells were assayed for O2 production in the presence of various concentrations of an equimolar mixture of mAbs 32 and 22. (B) cumulative O2 as a function of the concentration of the mAb 32/22 mixture at 6 (○), 40 (□), and 80 (△) min. O2 production triggered by various concentrations of mAb 197 (C, □) or by mAb 32-FcyRI complexes cross-linked by mAb 22 (C, □) is expressed as the amount of reduced cytochrome (Cyt) c accumulated by 6 (filled symbols) and 60 (open symbols) min after stimulation. Neither mAb 32 or 22 when added alone nor the Fab'2 fragment of mAb 197 was stimulatory (not shown). Values obtained in the absence of cross-linking mAb were subtracted.
the IgG2a subclass, it binds FcγRII both as a ligand through its Fc region and as an antibody to an epitope outside of the ligand-binding site. Fig. 3C shows that O2− generation was stimulated by mAb 197 alone and that the stimulation was bimodal over the same range of concentrations which gave transient and continuous stimulation by mAbs 32/22 (Fig. 2B) or mAb 32 plus second antibody (Fig. 1B) or plus mAb 22 (Fig. 3C). In contrast, oxidase activity was not triggered by the F(ab′)2 fragment of mAb 197 (not shown).

Collectively, these results demonstrate that transient activation of the NADPH oxidase of O2− is a function of the concentration, but not the type, of FcγRII cross-linking antibody. Functional similarities among the stimulatory concentrations may be an indication that quantitative similar stimulatory units are being formed.

Resumption of O2− Production in Prestimulated Cells—To activate a transient O2− burst, mAb 32-FcγRII complexes on A12.13 cells were cross-linked with 10 μg/ml goat F(ab′)2. Following the cessation of O2− production, oxidase activity was restimulated with the addition of phorbol 12-myristate 13-acetate or aggregated IgG1. Second reagent stimulation reactivated the oxidase system in the prestimulated cells to levels comparable to those of the control cells (Fig. 4). This shows that neither the phorbol 12-myristate 13-acetate nor surface receptor-mediated stimulation of the oxidase was blocked in cells that had been triggered by high concentrations of cross-linking antibody. Therefore, cessation of O2− does not involve a generalized inhibition of the oxidase.

Effect of KCN on Early Cessation of O2− Generation—Since O2− generation requires ATP, we measured the kinetics of O2− release from mAb 32-precoated cells that were stimulated with second antibody in the presence or absence of KCN. The results (Fig. 5) indicated that maximal levels of O2− release were not altered by the presence of KCN. More importantly, reoxidation of the cytochrome c that was reduced during the early burst of oxidase activity or during low but prolonged oxidase activity was effectively blocked in assays containing KCN. Thus, inclusion of KCN in the assay prevented reoxidation of reduced cytochrome without affecting its rate of reduction. The interval over which reduced cytochrome c was stabilized was sufficient to make cytochrome c reduction in the presence of KCN a quantitative measure of cumulative O2− levels.

Comparison of Binding and Stimulation by mAbs 32/22 or

![Fig. 4. Restimulation of NADPH oxidase activity following the cessation of O2− production induced by cross-linking of FcγRII.](image)

![Fig. 5. Quantitation of O2− generation: effect of KCN on the rate and amount of cytochrome (Cyt) c reduction.](image)

197—We tested cells on which FcγRII had been cross-linked for the kinetics of O2− generation and the kinetics of the binding by mAb 197. This was carried out at various concentrations of the cross-linking mAb. Fig. 6, A–C, shows the results of mAb F-197 binding to the cells during stimulation of the oxidase. The three concentrations selected to stimulate the cells are from the high, low, and intermediate concentrations and give representative rates of binding and O2− release for those concentration ranges. In most cases, there was an apparently linear relationship between the two activities. The exception was that at very low concentrations of cross-linking mAbs, there was a delay in O2− production following the binding of mAb to the cells. Fig. 6D shows cumulative data from the binding and stimulation experiments in which mAb F-197 or mAbs F-32/22 had been used as cross-linking antibody. We show by these comparisons that (a) O2− generation ceased when there was no further accumulation of cross-linking antibody on the cell; and (b) that O2− generation continued as long as mAb continued to accumulate on the cells.

In addition, we found that after cells had been incubated in cross-linking antibody for a period of time, the accumulation of the antibody on the cells stopped. This accumulation of antibody was measured for each concentration of cross-linking antibody in several experiments (Fig. 7). Our data indicated that the total amount of antibody that accumulated on the cells was dependent on the concentration of free antibody at the start of the incubation and presumably on the avidity of its binding to FcγRII. The data also show that concentrations of antibody in the high range saturated FcγRII-binding sites and that the tested low concentrations of mAbs were well below those required to saturate available receptors.

To establish whether there was any difference in the relative effectiveness of bound mAb 197 and mAbs 32/22 to stimulate the oxidase, we compared the total amount of accumulated cell-associated mAb with the total amount of accumulated O2−. We found that over the range of FcγRII-saturating and -sub saturating concentrations of mAb 197 (Fig. 7), stimulation by this antibody produced a set ratio of total O2− evolved per unit of mAb bound (Table I). In the presence of a 500-fold excess of IgG, we found very little difference in the final ratio of O2− per unit of mAb bound. By contrast, increased concentrations of mAbs F-32/22 increased the
amount of mAb F-32 bound (Fig. 7) but decreased the amount of $O_2^-$ produced per unit of mAb F-32 bound (Table I).

To measure the effect of hIgG at different times during stimulation by mAb 197, cells were preincubated in a 500-fold excess of hIgG, and the kinetics of $O_2^-$ generation and mAb binding were determined. $O_2^-$ generation was initially blocked (Fig. 8A) and the mAb 197-binding rate reduced (Fig. 8B) by hIgG. However, after 5 min, the amount of $O_2^-$ generation triggered per unit of bound mAb 197 in the presence of hIgG (Fig. 8C) approached that for mAb 197 alone. The $O_2^-$ generation and mAb binding of cells in the presence and absence of hIgG improved after 30 min. Since some mAb 197 would act as a monovalent ligand to the receptor Fc-binding site, the increase in production per unit of mAb 197 bound was presumably due to displacement of some monovalently associating antibody by the higher affinity configuration of mAb, bound initially through its F(ab')2 region. The decreased number of mAb 197 bound in the presence of hIgG was not due to an hIgG1-promoted internalization of FcγRI, as indicated by the increased binding of mAb 197 in the presence of hIgG1 (Fig. 8D).

TABLE I

| mAb concentration (µg/ml) | ratio bound mAb/nmol cytochrome c reduced | F-mAb 197 (Exp. 1) | F-mAb 197 (Exp. 2) | F-mAb 32/22* |
|---------------------------|------------------------------------------|---------------------|---------------------|--------------|
|                           |                                          | 10.1 ± 0.6          | 11.3 ± 0.2          | 18.0 ± 0.6   |
|                           |                                          | 2.5                 | 9.1 ± 0.3           | 10.8 ± 0.6   |
|                           |                                          | 1                   | 10.5 ± 0.2          | 15.9 ± 1.4   |
|                           |                                          | 0.5                 | 10.1 ± 0.1          | 12.8 ± 0.9   |
|                           |                                          | 0.25                | 6.7 ± 1.3           | 11.1 ± 1.0   |
|                           |                                          | 0.2                 | 9.1 ± 0.1           | [142,000 ± 9,000]  |
|                           |                                          | 0.1                 | 11.0 ± 0.6          | [117,000 ± 5,000]  |
|                           |                                          | 0.2 + hIgG*         | 7.8 ± 0.6           | [117,000 ± 5,000]  |

*Concentrations given are for F-mAb 32 in the equimolar mAb 32/22 mixture.

Numbers of molecules of bound mAb F-197 were estimated by comparing values obtained in Experiment 3 to the MFI of beads conjugated with known amounts of FITC. Molecular concentrations were determined by correcting values with the FITC/protein ratio of mAb F-197 as described in Ref. 37.
Therefore, it is probable that mAb 197 triggers oxidase activation by cross-linking FcγRI through the binding of Fc and one or both Fab domains. We suggest that Fc and one Fab domain of mAb 197 were insufficient to trigger oxidase activity when present (0) or absence (0) of hIgG. Some cells were incubated with the same high affinity as the murine IgG2a Fc domain to initiate oxidation of second antibody was 0.25 pg/ml (Fig. 9C). The antibody was not found in the detergent-insoluble fraction. As shown in Fig. 9A, 5 μg/ml second antibody triggered a transient burst of O₂⁻ and a transient association of detectable levels of bound mAb F-32 with cell cytoskeletons (Fig. 9C). Dissociation of mAb 32 from the detergent-insoluble material occurred when 72% of total bound mAb was still cell associated (Fig. 9B). The amount of FcγRI association with the cell cytoskeleton was considerably less when the concentration of antibody was 0.25 μg/ml (Fig. 9C). This concentration was less stimulatory to the cells (Fig. 9A). The results show that the transient O₂⁻ burst preceded cytoskeletal dissociation and coincided with cytoskeletal association of FcγRI-bound mAb F-32.

FIG. 8. Inhibition by hIgG of mAb 197-stimulated O₂⁻ generation. Cells were incubated with mAb F-197 in the presence (closed symbols) and absence (open symbols) of 200 μg/ml hIgG in a standard O₂⁻ assay. Samples taken at the indicated times were assayed for reduced cytochrome (Cyt) c (A) and cell-associated mAb F-197 (B). (C) inhibition of O₂⁻ release is expressed as the amount of cell-bound mAb F-197 (MPI) for each nmol of cytochrome c reduced in the presence (0) or absence (0) of hIgG. Some cells were incubated with F-mAb 32 at 4 °C for 60 min after they had been incubated with hIgG in the O₂⁻ assay above but without mAb 197. The presence of hIgG caused only a 5% decrease in surface FcγRI (not shown). Results are the means of triplicate cultures.

Cross-linking Antibody-dependent Association with Cell Cytoskeleton—Another phenomenon associated with receptor-mediated transient activation of the oxidase pathway is the association of receptors with the cell cytoskeleton (9). To determine whether FcγRI became associated with the cell cytoskeleton, we measured the amount of FcγRI in the detergent-insoluble fraction of lysed cells on which the receptors had been cross-linked. Fluorescein-labeled mAb 32 was bound to FcγRI and then cross-linked with second antibody. FcγRI that bound mAb F-32 but were not cross-linked by second antibody were not found in the detergent-insoluble fraction. As shown in Fig. 9A, 5 μg/ml second antibody triggered a transient burst of O₂⁻ and a transient association of detectable levels of bound mAb F-32 with cell cytoskeletons (Fig. 9C). Dissociation of mAb 32 from the detergent-insoluble material occurred when 72% of total bound mAb was still cell associated (Fig. 9B). The amount of FcγRI association with the cell cytoskeleton was considerably less when the concentration of antibody was 0.25 μg/ml (Fig. 9C). This concentration was less stimulatory to the cells (Fig. 9A). The results show that the transient O₂⁻ burst preceded cytoskeletal dissociation and coincided with cytoskeletal association of FcγRI-bound mAb F-32.

DISCUSSION

FcγRI-specific mAbs Trigger Oxidase Activity—The ability of antibodies to cross-link FcγRI has been defined by the induction of functions known to be triggered by multivalent FcγR ligands. The manner in which they interact with FcγRI to initiate O₂⁻ production has been determined using three different anti-FcγRI mAbs alone and in combination and by examining cells incubated with mAbs lacking specific domains required for cross-linking or in the presence of blocks of the receptor-binding sites. Binding by the Fab'2 of mAb 197 is insufficient to trigger O₂⁻ generation. In addition, hIgG1, which binds through its Fc domain to the ligand-binding site with the same high affinity as the murine IgG2a Fc domain of mAb 197 (4), temporarily blocks mAb 197-triggered O₂⁻ production. Therefore, it is probable that mAb 197 triggers oxidase activation by cross-linking FcγRI through the binding of Fc and one or both Fab domains. We suggest that Fc and Fab binding probably spans at least two receptors thereby linking numbers of FcγRI into a stimulatory receptor cluster. HlgG1 also delays mAb 197-dependent FcγRI internalization, additional evidence of receptor clustering (10) in the presence of this antibody. The interpretation that mAb 197 spans at least two receptors through its Fab and Fc binding is consistent with that of Kurlander (11) and MacIntyre et al. (12) who show evidence of whole antibody bridging of surface proteins to FcγRs on the same cell.

That the involvement of the natural ligand-binding site per se is not necessary for O₂⁻ stimulation can be seen in the stimulation of cells by the mAbs 32/22 mixture, which bind epitopes outside of the ligand-binding site. With regard to the stimulatory combination of mAbs 32/22, we have found that neither mAb 32 nor 22 alone stimulates O₂⁻ generation and that mAb 32 alone does not promote FcγRI association with the cell cytoskeleton or internalization. Each of the two

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antibodies, 32 and 22, is probably capable of spanning two receptors, as suggested by their ability to stimulate \(O_2^\cdot\) production and internalization\(^2\) when both are present. It has been assumed that stimulation of cell response through surface receptors involves a change in the conformation of the receptor which is transmitted to the cytoplasmic domain, initiating signaling events (6, 13). FcγRII may undergo a conformational change but that appears to be independent of ligand occupation and is supplantated or initiated by receptor aggregation.

Cross-linking Antibodies Trigger an Activation/Deactivation of the NADPH Oxidase—As expected from reports published previously on the \(O_2^\cdot\) response triggered by multivalent ligands such as opsonized particles (6), immune complexes (3), or aggregated hlgG1,\(^1\) low concentrations of cross-linking antibodies stimulated slow but continuous \(O_2^\cdot\) production for 30–80 min. However, when \(O_2^\cdot\) generation was triggered by high concentrations of cross-linking antibodies, a substantial but brief (<3 min) \(O_2^\cdot\) response was the rule. The rapid burst followed by an abrupt cessation suggested that a high rate of oxidase activity was terminated by an oxidase deactivation. If receptor cross-linking activates FcγRII to signal the oxidative response for the interval that the receptors are “occupied,” the apparent deactivation could have been due to exhaustion of oxidase substrate, depletion of available oxidase, or a generalized inhibition of the oxidase pathway. The following evidence suggests that this is not the case.

(a) Regardless of the total output by the oxidase (i.e. ~3 nmol of cytochrome c or 30 nmol/10\(^6\) cells) cessation occurred during the burst. Therefore, cessation is probably not the result of oxidase substrate depletion. In addition, there was no evidence of any inhibitory effect of KCN on the kinetics of oxidase activation/deactivation. Thus, it was apparent that we had not altered cytoplasmic ATP replenishment or NADPH levels, which may have inadvertently produced an apparent deactivation.

(b) Termination of oxidase activity took place in cells with demonstrably greater oxidative capacity. Therefore, cessation took place in the presence of additionally available but apparently unactivated oxidase molecules and was not due to oxidase exhaustion or desensitization.

(c) Additional stimulatory reagents reinitiated \(O_2^\cdot\) production by cells that had undergone an FcγRII-mediated oxidative burst. These agents included phorbol 12-myristate 13-acetate, which would be expected to activate protein kinase C (14–16), and aggregated IgG1, which would be expected to stimulate through the signal transduction pathways common to oxidase-signaling surface receptors. We have no direct evidence that the same molecules of oxidase deactivated by the first stimulus were reactivated by the second stimulatory reagents. However, resumption of oxidase activity in these cells indicated that cessation of \(O_2^\cdot\) generation was not due to a generalized inhibition of the NADPH oxidase system.

**Transient Oxidase Activation during Cluster Formation**—A comparison of the rates of binding of mAbs 197 and 32/22 to the rates of \(O_2^\cdot\) generation revealed that at saturating concentrations, the time required for maximal accumulation of the mAbs on the cell coincided with the cessation of \(O_2^\cdot\) production. At subsaturating concentrations of cross-linking mAbs, \(O_2^\cdot\) production continued over the same prolonged interval as mAb binding. The initial lag in production seen only in the presence of the lowest concentrations of cross-linking antibodies may have been the result of a low rate of recruitment of receptor di- or trimers into larger stimulatory clusters. This evidence strongly suggests that continued \(O_2^\cdot\) production is sustained by the continued binding of antibody which probably causes continued de novo formation of stimulatory receptor clusters. Moreover, similarities in the rates of \(O_2^\cdot\) production and of presumed cluster formation suggest a quantal relationship between activated receptors and the activation of the oxidase. Significantly, \(O_2^\cdot\) production ceased when cross-linking mAb binding ceased. This indicates that the activation event is probably transient.

Some published accounts of the deactivation of oxidase activity initiated through surface receptors (17–20) show that deactivation is rapid but do not indicate the state of the receptors when oxidase activity terminated. In some reports, deactivation has been attributed to rapid loss of ligand from ligand-receptor complexes followed by a putative reversion of the receptors to the nonstimulatory configuration. However, our experiments with cross-linkers of FcγRII indicate that oxidase activation is terminated in spite of the continued cross-linked state of the receptor. Furthermore, termination occurs prior to cluster internalization.\(^4\) Therefore, continued occupancy of the FcγRII by cross-linking antibody does not dictate continued oxidase activation.

**Novel Response to mAb 197**—When binding equilibrium was reached, the high concentrations of cross-linking mAbs had saturated FcγRII, whereas the subsaturating concentrations had bound to an extent that reflects the concentration of available antibody and its avidity for the receptors. An unusual finding was that when one of the cross-linking antibodies, mAb 197, had accumulated to binding equilibrium, the cells had produced a constant ratio of \(O_2^\cdot\)/mol of bound antibody. The ratio was the same regardless of the final amount of cell-associated mAb. A constant stoichiometry at equilibrium was not the case for the mAb 32/22 mixture, which triggered the production of decreasing total amounts of \(O_2^\cdot\)/mol of bound mAb as saturating concentrations were approached. The difference may be due to the fact that mAbs 32/22 require twice the specific binding events of mAb 197 to produce a stimulatory combination, and the production of such a stimulatory combination may be less favored at high mAb 32/22 concentrations.

The reasons for bound mAb 197 triggering amounts of \(O_2^\cdot\) production in proportion to the amount of mAb bound, without any apparent inhibitory effect by saturating concentrations of the antibody, are more subtle. We believe that the higher avidity of the mAb for FcγRI when it is bound through both its Fc and Fab domains may well prevent sustained monovalent Fc or Fab domain binding. If sustained monovalent binding is prevented, then excess mAb would be unable to block cross-linking and stimulation. In support of this explanation is the finding that hlgG1, which has the same affinity (21, 22) for the receptor ligand-binding site as the Fc domain of murine IgG2a (4), only transiently blocks the ability of mAb 197 to stimulate cells. It is possible that Ab 197 is displacing the block. Thus, stimulation in the presence of the monovalent ligand for the Fc-binding site supports our conclusion that the multivalency of mAb 197 increases its apparent affinity for that site beyond that of monomeric Fc ligands and enables the antibody to cross-link most of the receptors. This phenomenon of rapid displacement from surface receptors of high affinity monomeric (more dissociable) ligands by multivalent (less dissociable) ligands has been described previously (23, 24).

**Rate Versus Occupancy Theories of Receptor Activation**—There are two theoretical models of receptor activation for signaling cell response. The occupancy theory holds that receptors are in an activated state for the duration of ligand occupancy and that this state promotes sustained activation of cell response. Therefore, at equilibrium binding, the rate
of cell response should be highest. In the early 1960s, Paton proposed a rate model in which a rate of response would be determined by the rate of formation of ligand-receptor complexes (25, 26). In this case, the ligand-binding event would trigger one quantum of excitation before inactivation of the ligand-receptor complex. Thus, the rate of response would be dependent on the rate of de novo formation of complexes. In a strict interpretation of a quantum of excitation, each activated receptor would elicit a set unit, or quantum, of response regardless of the number of receptors involved or when they became activated.

That such a quantal relationship between cross-linked, and therefore activated, FcγRI and oxidase activity may exist is suggested by two lines of evidence. First, there was an apparently constant linear relationship between the rate of accumulation of cross-linking mAbs 32/22 or 197 on the cells and the rate of accumulation of O2−-reduced cytochrome c. This would be expected if accretion of cross-linking ligands produced some number of newly activated FcγRI, each of which made a constant unitary contribution to signaling. Second, mAb 197 was unique in the constant stoichiometry between the mol of accumulated mAb 197 on the cells and mol of O2− produced regardless of the concentration of free mAb 197. This would be expected only if (a) the avidity of the cross-linked configuration of whole mAb 197 supported a constant and high ratio of cross-linked to non-cross-linked receptors, and (b) each mole of mAb 197 bound in the stimulatory configuration triggered a constant or quantal amount of oxidase activity.

The rapid reversibility of oxidase activation has been shown by numerous investigators (17–20, 27–29). Moreover, oxidase activation through the receptor for fMLP is transient (27, 29–32) and possibly quantal (30). In the case of the fMLP receptor, where monovalent ligand interacts with monovalent receptor, we would expect constant ligand-related responses because each receptor would be expected to make a unitary contribution. It was not predicted that FcγRI aggregates of possibly varied size might also promote responses that are proportional to ligand binding and, in the case of mAb 197, stoichiometric. Collectively, their results and ours suggest common rules governing ligand-receptor performance and suggest that, clustered or not, each activated receptor may signal in a unitary manner.

Transient Association with the Cell Cytoskeleton—Our experiments revealed a transient association of cross-linked FcγRI with the cell cytoskeletons of A2113 cells. At concentrations of cross-linking antibody saturating for the receptors, there was an apparently asynchronous association and then dissociation of cross-linking antibody from the detergent-insoluble material. This dissociation appears to follow rather than coincide with cessation of oxidase activity. When FcγRI were cross-linked more slowly so that there would be a continuous de novo receptor activation (as indicated by continued O2− production), association was so brief that there was little cytoskeletal associated FcγRI at any one instant. This lack of accumulation of postactivated receptor on cytoskeletal actin gives some indication of the transience of this processing step. Actin association following signaling through FcγRI consistent with the postsignaling processing events described for the FMLP receptors of neutrophils (9, 33–36).

Other Considerations Regarding Activation and Processing—Our observations of an apparent quantum of response argue that FcγRI is a rate receptor but do not reveal the molecular details about what terminates the activating event. The strict interpretation of the rate model presented by Van Haastert (13) defines a transient activated state as inherent to the signaling step and independent of subsequent processing steps. If cross-linked FcγRI continued to signal until bound through an actin-binding protein to the cell cytoskeleton, this step could act to terminate signaling and to determine the duration of each quantum of signal. Jesaitis and colleagues (33, 34), describing the processing of the FMLP receptor, have shown some evidence favoring this possibility. The molecular events underlying the presumed signal termination of the FMLP receptor have yet to be elucidated, but, given the similarities with the FcγRI, the results of such studies may well have broad application.

Summary—Cross-linking of FcγRI by three different cross-linking strategies produced an activation then a deactivation of the NADPH oxidase pathway. Our results favor an explanation for deactivation which describes the relationship between activated FcγRI and activated oxidase as transient, dependent upon the continued formation of activated FcγRI for sustained oxidase activation, and one that produces a set quantum of O2− for each cross-linked FcγRI.

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