Monoclonal Antibodies to Fe Receptors for IgG on Human
Mononuclear Phagocytes

ANTIBODY CHARACTERIZATION AND INDUCTION OF SUPEROXIDE PRODUCTION IN A MONOCYTE
CELL LINE

We have utilized monoclonal antibodies against the two IgG Fe receptors (p40 and p72) of U937 cells to
stimulate the release of superoxide. The mononuclear antibody (mAb) specific for p40 (IV3) has been
described elsewhere. A murine IgGl mAb specific for the high affinity p72 Fe receptor (designated mAb FeR32
or simply mAb 32) bound to the same p72 precipitated

**Monoclonal Antibodies to**

Cells, but not to granulocytes or lymphocytes. U937

by Sepharose-human IgG as shown by preclearing ex-

periments and by identical isoelectric focussing pat-

terns. Binding of mAb 32 to p72 was independent of

the Fe region of the antibody since Fab' fragments of

mAb 32 affinity adsorbed p72. The binding of both

mAb 32 and human IgG1 to the intact U937 cell was

not reciprocally inhibitory, indicating that mAb 32
does not interfere with the ligand binding site of p72.
mAb 32 bound to human monocytes, U937, and

HL60 cells, but not to granulocytes or lymphocytes. U937

cells cultured in \( \gamma \)-interferon and 1,25-dihydroxycholecalfi
erol generated superoxide when incubated with

mAb 32 or IV3 followed by cross-linking with F(ab')2

anti-murine Ig. Incubation with mAb 32 or IV3 alone

or with 3 of 5 other anti-U937 mAbs cross-linked with

anti-murine Ig did not result in superoxide generation.

Immune complex-mediated superoxide production was

inhibited 80% by IgGG, but not by mAb 32 or IV3.

Two distinct classes of IgG Fe receptors have been described on human monocytes and the human monocyte ceil line U937

(1). One is a 72-kDa sialoglycoprotein (p72) with high affinity

\( K_c = 10^{-10} \) M \(^{-1} \) for monomeric human IgG1 and IgG3 and

for murine subclasses IgG2a and IgG3 (2,4). The other recep-

tor is a 40-kDa molecule (p40) which shows relatively low

affinity for monomeric IgG (1,5). p40 has been defined by its

ability both to form rosettes with erythrocytes coated with

murine IgG1 (1) and to bind aggregated murine IgG2b at low

ionic strength (5). In addition, a mononuclear antibody (IV3)

has been prepared which binds to the 40-kDa receptor and

inhibits ligand binding (1). This receptor is present not only

on mononuclear phagocytes, but on human platelets, neutro-

phils, eosinophils, and B cells (6,7). These two receptors on

human monocytes have been shown to mediate anti-T3-in-

duced human T cell mitogenesis by distinct subclasses of

murine IgG. The 72-kDa Fe receptor mediates murine IgG2a

anti-T3-induced stimulation, whereas the 40-kDa Fe receptor

mediates murine IgGl anti-T3-induced T cell mitogenesis (1).

Based upon their distinctive affinities for murine IgG subclasses,
p72 and p40 are thought to be the human homologues of

murine macrophage Fe receptor I and Fe receptor II specific

for murine IgG2a and IgG2b/1, respectively (8,9). Although

not present on monocytes or U937 cells, a third class of IgG

Fe receptors has been described on human neutrophils and

cell null cells (10,11).

In an effort to study in detail the structure and function of

these two mononuclear phagocyte Fe receptors, we have

sought to develop monoclonal antibodies against each of them.

Herein we detail the development and characterization of

a monoclonal antibody specific for the 72-kDa high affinity Fe

receptor and show that cross-linking of monoclonal antibodies

interacting with either the 72-kDa or the 40-kDa receptors

results in superoxide production by U937 cells.

**MATERIALS AND METHODS**

**Chemicals and Reagents—**Cytochrome c Type VI, superoxide dismutase, peptatin, chymostatin, leupeptin, antipain, rabbit muscle actin, and phenylmethylsulfonyl fluoride were purchased from Sigma; dextran T500, Ficoll-Hypaque, Sepharose 4B, CNBr-activated Seph-

arose, and Protein A-Sepharose CL-4B from Pharmacia; tetanus
toxin, octyl-\( \beta \)-D-glucopyranoside (octylglucoside), and papain from Behring Diagnostics; human anti-tetanus toxin antibody (Hyper-Tet) from Cutter Laboratories, Berkeley, CA; chloroglycouril from Pierce Chemical Co.; carrier-free Na\(^{23} \) (IMS.300) from Amersham; cyto-

chalin B from Aldrich; goat F(ab')2 anti-murine Ig (anti-mlg'),

both fluorescein isothiocyanate-conjugated (FITC) and unconjugated,

from Cappel, West Chester, PA, unless indicated otherwise; RPMI 1640 from GIBCO and from K C Biologicals, Lenexa, KS; fetal bovine serum from Sterile Systems, Logan UT; and a mixture of low molec-

ular weight markers from Bio-Rad. Recombinant \( \gamma \)-interferon, titers for which are expressed in International Reference Units (IRU) based on the value of National Institutes of Health Standard Gg 02.5-301-

The abbreviations used are m, murine; h, human; FITC, fluorescein isothiocyanate; KRPglc, Krebs-Ringer phosphate buffer with glucose; PBS phosphate-buffered saline; mAb, monoclonal antibody; IPN-\( \gamma \), \( \gamma \)-interferon; BSA, bovine serum albumin; PAGE, polyacryl-

amide gel electrophoresis; 1,25(OH)\(_2\)D, 1,25-dihydroxycholecalciferol.

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amide gel electrophoresis; 1,25(OH)\(_2\)D, 1,25-dihydroxycholecalciferol.

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530, was kindly donated by Genentech, South San Francisco, CA. 1,25-Dihydroxycholecalciferol (1,25(OH)2D3) was a gift from Hoffmann-La Roche. Other chemicals were of analytical grade and were obtained commercially.

Nonidet P-40 lysis buffer contained 1% Nonidet P-40, 110 mM NaCl, 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml chymostatin, 10 μg/ml leupeptin, and 10 μg/ml antipain in 20 mM Tris buffer, pH 7.1. Krebs-Ringer phosphate buffer with glucose (KRPglc) consisted of 133 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 1 mM CaCl2, 4.3 mM glucose in 10 mM sodium phosphate buffer. The buffer was buffered to pH 7.0 using 20 mM phosphate buffer, pH 7.0. PBS-K contained 130 mM NaCl and 5 mM KCl in 10 mM phosphate buffer, pH 7.4. Antibodies—The monoclonal antibody against the high affinity Fc receptor (herein designated mAb12) was prepared as follows. A partially purified detergent lysate of the high affinity Fc receptor from U937 cells was obtained in a manner similar to a published method (12) by lysing U937 cells in 1% Nonidet P-40 buffer, pH 7.4. Krebs-Ringer phosphate buffer with glucose (KRPglc) consisted of 133 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 1 mM CaCl2, 4.3 mM glucose in 10 mM sodium phosphate buffer. The buffer was buffered to pH 7.0 using 20 mM phosphate buffer, pH 7.0. PBS-K contained 130 mM NaCl and 5 mM KCl in 10 mM phosphate buffer, pH 7.4. Antigens—Human granulocytes were obtained from the peripheral blood of normal donors by separation from mononuclear cells on Ficoll-Hypaque, sedimentation of erythrocytes using 3% dextran in PBS, and, finally, hypotonic lysis of residual erythrocytes. Preparations were 98% granulocytes. Mononuclear cells were obtained by Ficoll-Hypaque separation. Cells of the Daudi, Raji, Molt4, Jurkat, and J774 lines were maintained in continuous culture as described (3, 14). Cells used in the Dartmouth laboratories were obtained from the American Type Culture Collection; sources of the cells used in Rochester have been described (1, 3, 9). All cells were cultured in PBS prior to use and were >95% viable when tested by trypan blue exclusion. Radiolabeling and Affinity Adsorption—Cells were surface-radiodinated by the chloroglycouril method (27). 0.7 ml of cells in PBS at 14.5 X 106/ml were incubated with 1 μCi of 125I for 30 min at 0°C in a scintillation vial coated with 5 μg of chloroglycouril. The reaction was quenched, and the cells were washed three times with 5 ml PBS-K in PBS. The cells were then lysed in Nonidet P-40 lysis buffer for 30 min at 0°C. Cell nuclei and other insoluble material were pelleted by centrifugation at 7800 X g for 20 min.

SDS-PAGE and Isoelectric Focusing—Sepharose-125I anti-mlg (35 μl) was incubated with monoclonal antibody in immunofluorescence assay using an Amicon YM-10 filter and a Minicon apparatus to 0.5 ml, and emulsified with an equal volume of Freund's adjuvant, either complete for the first injection or incomplete for subsequent ones. The emulsion was injected periocularly four times at roughly 4-week intervals, the last two immunizations using antigen derived from U937 cells cultured 72 h in IFN-γ, 100 RIU/ml, to increase the yield of Fc receptor (14). Five days following the last immunization, the splenocytes were fused with cells of the NS-1 myeloma line by standard techniques (15, 16). Supernatants of the hybrids were screened for their ability to bind to U937 cells by an indirect immunofluorescence assay using a flow cytometer. Chosen hybrids were cloned by limiting dilution, and expanded either in culture or in ascites fluid. The protein carboxylic acid of the urea-containing O'Farrell sample buffer, and 12857.
Mannheim), and were washed a final three times. Stained cells were analyzed in Rochester on an EPICS C flow cytometer (Coulter EPICS Division, Hialeah, FL) or at Dartmouth on an Ortho 50H Cytofluorograf using argon lasers at 300 or 500 milliwatts of power. Green fluorescence was collected through a 525 nm bandpass filter on 10,000–50,000 cells gated for low angle light scatter (to exclude erythrocytes, platelets, dead cells, and debris) and 90° light scatter (to distinguish monocytes or neutrophils from lymphocytes) (31). The 90° light scatter signal characteristic of monocytes was determined by separately staining an aliquot with anti-monocyte antibody Leu-12858 M3. Based on these data, gates for 90° light scatter were adjusted so that the green fluorescence signal of monocytes and lymphocytes in mononuclear cell suspensions could be separately collected. Green fluorescence was collected as a logarithmic (Rochester) or linear (Dartmouth) signal.

The mean fluorescence channel from each experiment (Rochester) was converted to a linear scale using a factor based on the observation (using calibrated microspheres) that each increase of 25 channels in a 256-channel log scale equals a doubling of fluorescence intensity. Calibrated fluorescent microspheres (Coulter EPICS Division, Hialeah, FL) were run each day at the same settings. Mean fluorescence intensity data were corrected for instrument sensitivity by dividing by the relative fluorescence intensity of microspheres run on that day. The fluorescence gain was changed for certain experiments to keep the signal intensity on track. Calibration of the flow cytometer for these experiments was performed by adjusting fluorescence gain to ensure equal fluorescence intensity for calibrated microspheres each day.

**Superoxide Generation Assay**—1.5 × 10⁶ U937 cells grown for 72 h in IFN-γ (100 IU/ml) and 10 nM 3-(OH)D, were incubated in 60 μl of KRPgc containing various monoclonal antibodies at 5 μg/ml (if purified Ig) or at a 1:6 dilution (if supernatant from cloned hybrids) for 15–30 min at 4 °C. The cells were sedimented by centrifugation, resuspended in 50 μl of KRPgc and then transferred to a cuvette containing 0.95 μl of KRPgc and 100 nm of ferricytochrome c at 37 °C. Additional reference cuvettes contained the above plus 20 μg of superoxide dismutase. With λmax 550 nm in a Varian DMS 100 double-beam spectrophotometer at 37 °C (32, 33). The rate of superoxide dismutase-inhibitable cytochrome c reduction was calculated from the linear rate of absorbance change and the molar extinction coefficient for this reduction (21,000) (34). In some experiments, 50 μl of immune complexes (175 μg) were added to the cells after transfer to the warmed cuvette.

**RESULTS**

The strategy for the development of monoclonal antibodies against the high affinity 72-kDa Fc receptor had to deal with the observation that two of the four murine IgG subclasses, IgG2a and IgG3, bound with high affinity to this Fc receptor. Thus, any assay for Fc receptor binding would register all antibodies of these two subclasses. Our protocol, therefore, called for immunizing a mouse with partially purified Fc receptor from U937 cells, screening the hybrid supernatants for any Ig capable of binding U937 cells, eliminating from further consideration IgG2a and IgG3 antibodies, and evaluating the remaining antibodies for their capacity to immuno-precipitate 72-kDa surface molecules.

Twenty-nine supernatants contained Ig capable of binding U937 cells. Of these, 12 were of the IgG2a subclass, 1 was IgG3, 7 were IgG1, 2 were IgM, and 7 were either of mixed subclass or could not be typed. The supernatants of the cultures of cloned cells were then evaluated for their ability to bind to a 72-kDa cell surface molecule by an affinity adsorption assay. Sepharose-covalently linked to goat F(ab')2 anti-mouse Ig (Sepharose-anti-mlg) was incubated with a mixture of the supernatants and detergent lysates of surface-radioiodinated U937 cells. The eluates from washed adsorbent beads were analyzed by SDS-PAGE and autoradiography (not shown). Of the supernatants containing IgG2a and IgG3 anti-U937 antibodies adsorbed a 72-kDa molecule, as expected. Of the remaining supernatants, one IgG1 (designated mAb 32) was found capable of adsorbing a 72-kDa molecule and was chosen for further study. Of the remaining 6 IgG1 supernatants, 5 adsorbed a 110-kDa molecule and 1 adsorbed small amounts of a 72-kDa molecule.

Fig. 1 summarizes these observations. The autoradiograph shows the 72-kDa molecule purified from detergent lysates of radioiodinated U937 cells with mAb 32 (lane 3). The molecular weight of this molecule as determined on SDS-polyacrylamide gels is indistinguishable from the 72-kDa Fc receptor affinity adsorbed by Sepharose conjugated with ligand capable of interacting with the receptor. Thus, Sepharose-anti-mlg bearing RPC5, a murine IgG2a (lane 1), or Sepharose-human IgG (lane 4) both purify a 72-kDa molecule that has been shown to be the high affinity Fc receptor of U937 cells and human monocytes (26). An additional molecule of approximately 40 kDa was adsorbed by Sepharose-human IgG (lane 4). We have recently demonstrated this molecule to be a low affinity Fc receptor precipitated by mAb IV3 (lane 5) and present on other blood cells as well (see introduction to the text and Ref. 1). Purification of p40 requires an affinity adsorbent of high ligand to Sepharose ratio; hence, this molecule is recovered only from Sepharose-hlgG (7.6 mg/ml) adsorbents and not from Sepharose-anti-mlg sensitized with mlG2a (~1 mg/ml) (1).

Since mlG2a antibodies have not been found to bind to the U937 high affinity Fc receptor (3, 11, 35), and, since several mlG1 monoclonal antibodies have failed to immunoprecipitate the 72-kDa Fc receptor, it was inferred that mAb 32

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1. C. L. Anderson, unpublished observations.
bound the 72-kDa molecule as an antibody by the Fab portion of the antibody molecule rather than as a ligand by the Fc portion. To confirm this supposition, Fab' fragments of mAb 32 were prepared and were tested for their ability to precipitate p72. Lane 8 of Fig. 1 indicates that Sepharose-anti-mlg sensitized with these Fab' fragments of mAb 32 purifies the 72-kDa molecule, whereas Fab fragments of a ligand that ordinarily would bind and precipitate the receptor, pooled human IgG, do not (lane 7). The 72-kDa molecule purified by the intact IgG of mAb 32 is shown in lane 6 for reference.

Although the autoradiograph seen in Fig. 1 shows that the molecule purified by mAb 32 is of the same apparent molecular weight as the molecule purified by the ligands, two other methods were utilized to evaluate whether the molecules are identical. First, lysates of radioiodinated U937 cells were precleared with either an affinity adsorbent of mAb 32 or with a sham adsorbent (mlgG1 which does not bind or precipitate the Fc receptor). The precleared lysates were then tested for residual p72 by affinity adsorption with Sepharose-anti-mlg sensitized with mIgG2a, a ligand which binds this Fc receptor. Radioactive material bound to the adsorbents was analyzed by electrophoresis on SDS-polyacrylamide gels followed by autoradiography and densitometry. Comparing lanes 1 and 4 of Fig. 2, mAb 32 precleared 73% of the p72 subsequently purified by mIgG2a. The reciprocal experiment, preclearing with mIgG2a and then purifying p72 with mAb 32, indicates that 89% of p72 was precleared with mIgG2a (lane 3) compared with the mIgGl sham control (lane 2). Control experiments were performed in which the same reagent (mAb or ligand) was used both for preclearing and for subsequent purification. These showed the efficiency of preclearing to range from 81% when mAb 32 was used (lanes 2 and 5) to 93% when mIgG2a was used (lanes 1 and 6). We conclude, therefore, that mAb 32 binds to the same high affinity 72-kDa Fc receptor purified by ligand affinity adsorption.

The third method used to test whether the ligand and mAb 32 bind the same 72-kDa molecule was isoelectric focussing. P72 molecules purified as in Fig. 1 with mAb 32 and a mIgG2a were eluted from the affinity adsorbents with a urea-containing sample buffer and were analyzed by isoelectric focussing and autoradiography. An identical pattern of 10 distinct bands having isoelectric points ranging from pH 5–7 was seen in both lanes (Fig. 3, lanes 1 and 3). A similar although subtly distinct pattern of bands was seen in lane 5 analyzing the eluate from the IV3 affinity adsorbent which purifies only the 40-kDa Fc receptor. The eluate from Sepharose-human IgG which purifies both the 72- and 40-kDa molecules appears in lane 4 as a composite of the two isoelectric focussing patterns with some of the p72 molecules appearing dimmer than in lanes 1–3, most likely because the p72-ligand bond resists dissociation by urea unlike the p40-ligand bond (26) and unlike antibody-antigen interactions (lanes 1–3). Thus, these data further substantiate the identity of the 72-kDa molecules purified by both Fc receptor ligands and mAb 32.

Since IFN-γ enhances the expression of the high affinity Fc receptor (14, 35), we used indirect immunofluorescence and flow cytometry to examine the binding of mAb 32 to control and IFN-γ-treated U937 cells. Table I shows a 3-fold increase in binding of both mAb 32 and a murine IgG2a myeloma protein to IFN-γ-induced U937 cells. We also determined whether hIgG interferes with the binding of mAb 32 to the Fc receptor of U937 cells. As seen in Table I, hIgG significantly blocked the binding of mIgG2a to the Fc receptor of U937, while the binding of mAb 32 was unaffected. This suggests that mAb 32 binds to the 72-kDa Fc receptor at a site distinct from the ligand binding site.

We further quantified the ability of both mAb 32 and a ligand, in this case a human IgGl (hIgG1) myeloma protein (All), to inhibit the binding to U937 cells of either 12859-human

![Fig. 2. Affinity adsorption with ligand or mAb 32 after pre-clearing U937 lysates with ligand or mAb 32. Portions of a lysate of radioiodinated U937 cells were incubated with Sepharose-anti-mlg sensitized with several mAbs designated as "preclearing adsorbents" in the figure. The affinity adsorbents were centrifuged out of suspension and the supernatants were incubated with a second set of affinity adsorbents designated "final adsorbents." The eluates from the washed set of final adsorbents were processed as described in the legend to Fig. 1. A photograph of the set of 72-kDa bands, the only bands appearing on the autoradiograph, is displayed vertically. Densitometric tracings on paper of the autoradiograph bands were cut out and weighed; band density is expressed as mg per band. Percent depletion of p72 by the pre-clearing adsorbent was calculated by comparing lanes 2 and 3, 1 and 4, 2, and 5, and 1 and 6.](attachment:image)

![Fig. 3. Isoelectric focussing of p72 purified either with ligand or with mAb 32. A detergent lysate of radioiodinated U937 cells was incubated with Sepharose-anti-mlg sensitized with either murine IgG2a myeloma RPC5 (lane 1) or mAb 32 (lane 3), with Sepharose-human IgG (lane 4); or with Sepharose-anti-mlg sensitized with mAb IV3 (lane 5). The radioactivity bound to the washed immunoadsorbents was eluted with an urea-containing sample buffer and was analyzed by isoelectric focussing and autoradiography. The pH gradient is shown in the left margin. Lane 2 analyzes a mAb still under investigation.](attachment:image)
TABLE I

Binding of mAb 32 to control- and IFN-γ treated U937 cells.

<table>
<thead>
<tr>
<th>First antibody</th>
<th>U937 without IFN-γ</th>
<th>U937 with IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No hIgG</td>
<td>hIgG</td>
</tr>
<tr>
<td>P3 (mIgG1)</td>
<td>39 ± 1</td>
<td>46 ± 4</td>
</tr>
<tr>
<td>mAb 32(mIgG1)</td>
<td>156 ± 4</td>
<td>15 ± 6</td>
</tr>
<tr>
<td>RPC5(mIgG2a)</td>
<td>158 ± 16</td>
<td>47 ± 2</td>
</tr>
</tbody>
</table>

FIG. 4. Double reciprocal binding of mAb 32 and human IgG1 to U937 cells. Under conditions of saturation and equilibrium, U937 cells were incubated at 4°C with (A) 125I-human IgG1 or (B) 125I-mAb 32 in the presence of varying amounts of unlabeled human IgG1 or mAb 32. Bound labeled antibody was separated from free by centrifuging the cells through oil and was quantified by counting the radioactivity associated with the cell pellets. Nonspecific binding measured in the presence of a great excess (>100-fold) of unlabeled antibody was subtracted from total binding to give specific binding. Percent inhibition, calculated as described under "Materials and Methods," was plotted versus the concentration of inhibitor protein. Nonspecific binding was 6–8% of total binding.

IgG1 (All) or 125I-mAb 32. As seen in Fig. 4A, 50% inhibition of 125I-hIgG1 binding was achieved with 10 nM hIgG1, whereas only 40% inhibition was seen with about 1 mM mAb 32, approximately a 100-fold difference in inhibitory capacities. The reciprocal experiment, seen in Fig. 4B, indicates that mAb 32 inhibited the binding to U937 cells of 125I-mAb 32 greater than 100 times more efficiently than the ligand hIgG1.

We conclude that the binding of mAb 32 to cells does not interfere with ligand binding to the Fc receptor binding site and that ligand binding does not inhibit mAb 32 binding.

The types of cells which bear the epitope recognized by mAb 32 were evaluated by indirect immunofluorescence and flow cytometry. Data from our laboratories at Dartmouth and Rochester were similar and are presented in Table II. It is apparent that mAb 32 binds to those cells which are known to bear the high affinity IgG Fc receptor, namely, U937, HLA, and monocytes. Lymphocytes were negative, as were the B cell lines Raji and Daudi, and the T cell lines Molt4 and Jurkat. The erythroblastic line K562, and the murine macrophage line J774, which bears both murine Fc receptor I and Fc receptor II, were negative as well. Some samples of neutrophils showed low level unimodal binding of mAb 32. The minor disparities in fluorescence intensity of specific cell lines between the two laboratories probably result from differences in experimental procedure and differences in the cell line subcultures. As illustrated in Fig. 5, it should be noted that, in spite of some overlap in the fluorescence intensity distribution of cells stained with mAb 32 and control antibodies, fluorescence intensity plots of all positive cell types indicated a unimodal binding with mAb 32. This suggests that the entire populations of HL60, U937 cells, and monocytes, rather than just a major subpopulation, were positive for binding of mAb 32.

We next examined the ability of mAb 32 to trigger superoxide production, a cellular response attributable to interaction with the 72-kDa high affinity Fc receptor on U937 cells. In preliminary experiments we learned that U937 cells could not be stimulated to produce superoxide with aggregated hIgG, immune complexes formed between human anti-tetanus and tetanus toxoid, or with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate. However, U937 cells which had been cultured for 72 h in IFN-γ and 1,25(OH)2D3 (conditions of Rigby and Guyre) produced superoxide upon incubation with immune complexes. In seven experiments, 175 µg/ml immune complexes induced these cultured U937 cells to produce 1.35 ± 0.70 O2−/min/106 cells.

A panel of mAbs was tested for their ability to induce superoxide production by these IFN-γ- and 1,25(OH)2D3-treated U937 cells. None of the mAbs listed in Table III, including mAb 32, was able to induce superoxide production following incubation with U937 cells (data not shown). However, prompt (maximum rates attained within 30 s) and sustained release of superoxide was achieved when a second cross-linking anti-mIg antibody (affinity-purified Fab'2 fragments) was added to cells previously incubated with mAb 32 (Table III). Similarly, the addition of anti-mIg to cells incubated with a ligand for the high affinity Fc receptor (a mlgG2a myeloma protein, RPC5) induced superoxide production. The same response was seen when anti-mIg was added to cells which had been incubated with IV3, the mAb directed against the low affinity Fc receptor for 40 kDa on U937 cells (1, 5-7). Equivalent results were obtained with purified IgG samples of either mAb 32 and IV3 or with supernatants from cloned hybrids producing mAb 32 and IV3, indicating that the superoxide produced is not due to a contaminating mlgG2a in the preparations. Neither the tissue culture medium itself nor the supernatant of two irrelevant mAbs together with an anti-mIg was able to induce superoxide production. Furthermore, Fab fragments of IV3 were also effective stimulators of superoxide production when cross-linked with an anti-mIg (data not shown). Two other mAbs with specificity for U937 cells, both IgG2b proteins, were capable of inducing superoxide production when cross-linked, namely AMI-2-23 which recognizes a 50–55-kDa molecule on induced U937 cells (16, 19) and MY7 (23), which binds to an undefined site on U937 cells. Four other mAbs, however, were incapable of inducing superoxide production. These include mAb MY23, a mlgG1 specific for a 50–55-kDa molecule on induced U937 cells (20); MAA, a mlgM which binds to an uncharacterized site of U937 cells (21); Gap 8.3, a mlgG2a protein with specificity for a 200-kDa surface molecule (22); and a mlgG2b anti-µ chain mAb (anti-Vk3b) which does not bind to U937 cells (18).

Immune complex-induced superoxide production by U937 cells.
The specific methods of this experiment varied slightly between the two laboratories, at Dartmouth and Rochester, and are detailed under "Materials and Methods." In brief, cells from various lines, peripheral blood mononuclear cells, and purified granulocytes were incubated first with either mAb 32 or mIgG2a and with control myeloma proteins of the mIgG1 (Dartmouth) or mIgG2b (Rochester) subclasses. The washed cells were then incubated with FITC anti-mIg, washed again, and analyzed by fluorescence intensity by flow cytometry. Results are expressed as mean fluorescence intensity in arbitrary units ± S.D.

| Cell type | Mean fluorescence intensity |  |
|-----------|-----------------------------|  |
|           | mAb 32 | Control | n | mAb 32 | Control | mIgG2a | n |
| U937      | 109 ± 21 | 25 ± 6 | 5 | 103 ± 48 | 76 ± 10 | 11 | 92 ± 20 | 6 |
| HL60      | 49 ± 12 | 23 ± 4 | 3 | 78 ± 18 | 16 ± 7 | 7 | 80 ± 26 | 3 |
| Monocytes | 67 ± 12 | 32 ± 6 | 4 | 113 ± 37 | 28 ± 11 | 8 |  |
| Lymphocytes | 7 ± 0.1 | 7 ± 0.4 | 4 | 10 ± 2 | 9 ± 3 | 2 |  |
| Neutrophils | 11 ± 1 | 8 ± 3 | 3 | 30 ± 4 | 23 ± 3 | 2 |  |
| Daudi     | 20      | 20     | 2 | 34 ± 3 | 33 ± 2 | 3 |  |
| Raji      | 34      | 32     | 1 | 18 ± 2 | 18 ± 2 | 3 |  |
| Molt4     | 15      | 14     | 1 | 22 ± 3 | 23 ± 3 | 3 |  |
| Jurkat    | 15      | 14     | 1 | 22 ± 3 | 23 ± 3 | 3 |  |
| K562      | 30      | 30     | 1 | 22 ± 3 | 23 ± 3 | 3 |  |
| J774      | 77      | 91     | 1 | 22 ± 3 | 23 ± 3 | 3 |  |

DISCUSSION

The development of monoclonal antibody against the high affinity Fc receptor for IgG of human mononuclear phagocytic cells was a particular challenge because two subclasses of murine IgG, IgG2a and IgG3, are ligands capable of binding with high affinity to this receptor (3, 4, 35). Thus, any assay for mAbs capable of binding to the receptor would register as positive all antibodies of these two subclasses. Our strategy was designed to deal with this obstacle, however, and we were successful in obtaining a monoclonal antibody of the IgGl subclass capable of binding this receptor at a site on the outer surface of the plasma membrane distinct from the ligand binding site. The data supporting this conclusion can be summarized briefly:

First, mAb 32 is of the IgGl subclass. This murine IgG subclass has been found incapable of binding to the high affinity Fc receptor (3, 4, 35). Of the seven IgGl mAbs with anti-U937 activity derived from the fusion described above, four of them absorbed only a 110-kDa molecule from lysates of surface-radioiodinated U937 cells. Thus, adsorption of the 72-kDa Fc receptor is not a general property of IgGl proteins. (Of the two remaining IgGl mAbs, one adsorbed small amounts of a 72-kDa molecule, and the other adsorbed both a 72-kDa and a 110-kDa molecule; these have not yet been further investigated.) Nevertheless, to eliminate the possibility that mAb 32 was a variant-IgGl which bound through its Fc region the Fc receptor, we tested the capacity of Fab' fragments of mAb 32 to adsorb the 72-kDa Fc receptor and found that binding occurs independently of the Fc portion of the mAb (Fig. 1).

Second, our data show that the 72-kDa molecule adsorbed by mAb 32 is the same molecule identified as the high affinity Fc receptor by several criteria previously described (26). Not only do the molecules appear identical by polyacrylamide gel electrophoresis in SDS (Fig. 1), but the isoelectric focussing patterns of the two molecules are the same as well (Fig. 3). The marked heterogeneity of charge of this molecule has been ascribed to terminal sialic acid residues (26). The preadsorption experiments shown in Fig. 2 also support the contention that the 72-kDa molecules bound by both ligand and mAb 32 are identical. Either ligand or mAb 32 is capable of removing the same 72-kDa molecule from detergent solution such that it is no longer available to the other for adsorption.

Third, the data indicate the mAb 32 binds to a site on the 72-kDa Fc receptor distinct from the site where ligand binds (Fig. 4 and Table I). This observation constitutes direct evidence that, in fact, mAb 32 is not binding to the receptor as a ligand, for if it were it should inhibit ligand binding. The capacity of mAb 32 to bind the ligand-occupied Fc receptor should prove useful in a number of circumstances involving detection of the receptor in the presence of ligand. To date, this has been impossible.

Fourth, it is quite clear from Table II that the only cells which bear the epitope recognized by mAb 32 are those which bear the 72-kDa high affinity Fc receptor, namely, monocytes.
monoclonal IgG2b control trophils of normal subjects show subtle evidence of induction by gating on forward and 90° light scatter. The fluorescence detection gain was set at 1400. The number for cells stained with mAb 32 microspheres. 256-channel histogram gated on forward angle light scatter (11,000 cells counted). The fluorescence detection gain was set at 1500 to bring the lymphocytes on scale.

2. Effect of human IgG on tetanus toxoid-antitoxin immune complex-mediated superoxide production. U937 cells (1.5 × 10^6 in 50 µl) were incubated with the indicated concentrations of either pooled human IgG (hIgG) or a human IgG1 myeloma protein (hIgG1) for 15 min at 0°C. The mixture was then added to 0.36 ml of warmed KRPglu containing 100 nmol of cytochrome c. After no more than 30 s, 50 µl of immune complex (175 µg) were added and the maximum rate of superoxide production was determined. Results are expressed as percent of control in which no inhibitors were added.

III. These results also show that both the high affinity Fc receptor (72 kDa) and the low affinity Fc receptor (40 kDa) are functional receptors, since mAbs directed against either receptor are capable of inducing superoxide production when

### Table III

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Molecule recognized</th>
<th>Source</th>
<th>Isotype</th>
<th>nmol O_2/min/10^6 cells</th>
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<tbody>
<tr>
<td>mAb 32</td>
<td>p72</td>
<td>Sup</td>
<td>IgG1</td>
<td>1.08 ± 0.19</td>
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<tr>
<td>RPC5</td>
<td>p72</td>
<td>Ig</td>
<td>IgG2a</td>
<td>1.16 ± 0.60</td>
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<tr>
<td>IV3</td>
<td>p40</td>
<td>Sup</td>
<td>IgG2b</td>
<td>2.00 ± 0.72</td>
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<td>p50-55</td>
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<td>IgG1</td>
<td>0</td>
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<tr>
<td>MAA</td>
<td>Undetermined</td>
<td>Ig</td>
<td>IgM</td>
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<td>IgG2b</td>
<td>0</td>
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<td>Ascs</td>
<td>IgG2a</td>
<td>0</td>
</tr>
<tr>
<td>AML-7-23</td>
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<td>Ig</td>
<td>IgG2b</td>
<td>1.11 ± 0.47</td>
</tr>
<tr>
<td>MY7</td>
<td>Undetermined</td>
<td>Ig</td>
<td>IgG2b</td>
<td>1.37 ± 0.42</td>
</tr>
</tbody>
</table>

Fig. 6 (left). Effect of human IgG on tetanus toxoid-antitoxin immune complex-mediated superoxide production. U937 cells (1.5 × 10^6 in 50 µl) were incubated with the indicated concentrations of either pooled human IgG (hIgG) or a human IgG1 myeloma protein (hIgG1) for 15 min at 0°C. The mixture was then added to 0.36 ml of warmed KRPglu containing 100 nmol of cytochrome c. After no more than 30 s, 50 µl of immune complex (175 µg) were added and the maximum rate of superoxide production was determined. Results are expressed as percent of control in which no inhibitors were added.

Fig. 7 (right). Effect of monoclonal antibodies on immune complex-induced superoxide production. U937 cells (1.5 × 10^6 in 50 µl) were incubated with the indicated concentrations of mAb for 45 min at 0°C. The mixture was then transferred to 0.95 ml of warmed KRPglu containing the same concentration of mAb, 100 nmol of cytochrome c, and 5 µg/ml cytochalasin B (to maximize the detected rate of superoxide production). Immune complex was added and superoxide production was determined and expressed as in Fig. 6.

**Fig. 5.** Fluorescence intensity of cells stained with mAb 32. Each panel displays histograms of fluorescence intensity versus cell number for cells stained with mAb 32 (shaded area) and murine monoclonal IgG2b control (solid line). Cells shown are lymphocytes (a), monocytes (b), and U937 cells (c). Monocytes and lymphocytes from a single suspension of blood mononuclear cells were identified by gating on forward and 90° light scatter. Panels a and b are 64-channel histograms (20,000 cells counted). The fluorescence detection gain was set at 1400. The scale of fluorescence intensity units was calibrated by fluorescent microspheres.

HL60 cells, and U937 cells. This correlation is further evidence that mAb 32 is directed against the high affinity Fc receptor. Neutrophils, according to the data of Table II, are the only other cells capable of binding mAb 32, but the extent of binding is so low as to be equivocal. Given observations that IFN-γ induces the expression of this high affinity Fc receptor on neutrophils (35), it is conceivable that the neutrophils of normal subjects show subtle evidence of induction of this receptor.

Although neither mAb 32 nor a monoclonal antibody directed against another Fc receptor class on U937 cells (IV3) was able to induce superoxide production when used alone, both were capable, when cross-linked with a second antibody, of triggering the release of superoxide. Furthermore, cross-linking a ligand (the murine IgG2 myeloma, RPC5) of the high affinity Fc receptor caused superoxide release (Table 1).
cross-linked with an anti-mIg. Although our data give no insight into the mechanism of superoxide triggering, presumably Fc receptors are being cross-linked just as with triggering by immune complexes (36, 37). Ligand occupation by the receptor appears to be unnecessary for triggering, similar to mast cell histamine release in response to anti-Fc receptor antibodies (38).

In our experiments, three of the other five mAbs directed against the U937 cell failed to induce superoxide when cross-linked with anti-Ig, and a sixth protein of the same subclass as IV3 but with no specificity for the U937 cell also failed to induce superoxide. The two exceptions which stimulated superoxide production were MY7 and AML-2-23, both IgG2b proteins purified from ascites fluid. Although all of the IgG2b anti-U937 proteins which we used resulted in superoxide stimulation, we think that the subclass of IV3 had nothing to do with its capacity to produce superoxide since Fab fragments of IV3 were as capable as the intact IgG of causing the effect (not shown). It is conceivable that enough contaminating mIgG2a was present in these IgG2b preparations to stimulate superoxide release by direct ligand binding and cross-linking. This is likely the case with AML-2-23 because Fab fragments which were shown to be capable of binding to U937 cells did not stimulate superoxide production upon cross-linking with anti-mIg (not shown). For MY7, however, we have not evaluated this possibility. Alternatively, the MY7 molecule may be associated with a superoxide-producing mechanism such that cross-linking MY7 triggers superoxide release. mAbs which are capable of triggering superoxide production have been described by other workers, although the identity of the molecules recognized by these mAbs has not been determined (39).

The failure of Gap8.3, an IgG2a protein which would be expected to bind as ligand to the high affinity Fc receptor, to stimulate superoxide production upon the addition of anti-mIg is noteworthy. One possibility for the failure is that binding of Gap8.3 simultaneously to both its antigen and the 72-kDa Fc receptor is adequate for maximum superoxide production. Attempts to reveal possible IV3 inhibition of superoxide production under conditions wherein the 72-kDa Fc receptor should be at least partially blocked by ligand were unproductive (Fig. 7). Complete blockade of the 72-kDa Fc receptor to prevent occupation by immune complexes is probably not possible using ligand (40), but will require a mAb with high affinity for the Fc receptor binding site.

All three of these possibilities for the failure of IV3 to inhibit are consistent with the observation shown in Figs. 6 and 7 that uncomplexed IgG is capable of inhibiting by 80% the induction of superoxide production by immune complexes. Similar preparations of IgG efficiently inhibit monomeric ligand binding to the 72-kDa Fc receptor (1, 3, 7). The failure of IgG, either human or murine, to inhibit more than 80% completely is most likely due to the efficiency with which immune complexes bind cells (40) and to the rapid off-rate of monomeric ligand from the 72-kDa Fc receptor (70% = 12 min) at 37 °C (3). Alternatively, another Fc receptor, unblocked by IgG and IV3, may be present on these induced cells, although the Fc receptor recognized by mAb 3G8 (10) was not found.4 While blockade of the 40-kDa Fc receptor with IV3 seemed not to diminish the immune complex-mediated response of these U937 cells, with other cell types, IV3 is capable of a marked inhibition of Fc receptor-mediated biological responses. For example, platelet aggregation induced by aggregated IgG is completely inhibited by IV3 (6), and the immune complex-mediated production of superoxide by granulocytes is markedly reduced by IV3 (1).

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Anti-Fc Receptor Superoxide Production