# MODULATION OF FC RECEPTORS OF MONONUCLEAR PHAGOCYTES BY IMMOBILIZED ANTIGEN-ANTIBODY COMPLEXES

## Quantitative Analysis of the Relationship between Ligand Number and Fc Receptor Response*

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Contact of animal cells with ligand-coated surfaces profoundly affects the functional activity of their plasma membrane receptors (1–7). For instance, monocytes and macrophages plated on surfaces coated with IgG containing antigen-antibody complexes show markedly reduced levels of binding and ingestion of IgG-coated erythrocytes (1–4). Our previous studies suggested that the extent of receptor modulation is dependent on the concentration of ligands on the substrate (cf. Fig. 3 in reference 2). Further work was needed to confirm this point, and more importantly, to determine whether the observed modulation of Fc receptors by immune complex-coated substrates is due to the functional inactivation of these receptors or to their physical removal from the nonadherent portions of the plasma membrane. The development of a monoclonal antibody against the trypsin-resistant Fc receptor (FcR)II[1] of mouse macrophages (8) has provided a unique reagent for probing this question. Using this monoclonal antibody we have found that FcRII is physically removed from the surface of macrophages maintained on immune complex-coated surfaces, and that there is a stoichiometric relationship between the number of IgG molecules bound to antigen on the substrate and the extent of FcRII modulation. In a companion paper (9) we show that diffusion is the mechanism of FcRII modulation. A preliminary report of some of these findings has been published (10).

## Materials and Methods

*Macrophages.* Resident peritoneal cells (24–36% macrophages) and thioglycollate broth-elicited peritoneal cells (72–86% macrophages) were obtained from NCS mice as described (2). Resident macrophages were cultured on plastic tissue culture petri dishes for 20 h, released with 15 mM Lidocaine (Abbott Laboratories, North Chicago, IL) in Eagle's minimum essential

* Supported in part by grants AI 19056, AI 14603, and AI 08697 from the National Institutes of Health.

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§ Recipient of Faculty Research Award FRA 205 from the American Cancer Society.

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; DNP, dinitrobenzene; E, sheep erythrocytes; E(IgG), E coated with anti-E IgG; FcR, receptor for the Fc portion of immunogobulin G; FcRII, trypsin-resistant FcR; HBSS, Hanks' balanced salt solution; hFBS, heat-inactivated fetal bovine serum; MEM, Eagle's minimum essential medium; PBS, phosphate-buffered saline with $Ca^{++}$ and $Mg^{++}$ ions; PD, PBS without $Ca^{++}$ and $Mg^{++}$; PLL, poly-L-lysine; RαDNP IgG, rabbit anti-DNP IgG.

medium (MEM) with Earle's salt solution, 10% heat-inactivated fetal bovine serum (hFBS) as described (11), washed, and used as described in Results. Where indicated resident macrophages were trypsinized in suspension as described (2) before plating.

*Preparation of Coverslips.* 12-mm diameter glass coverslips (SGA Scientific, Inc., Bloomfield, NJ) were prepared and coated with poly-L-lysine (PLL) (mol wt ~70,000; Miles Laboratories Inc., Elkhart, IN), dinitrophenyl (DNP) (Eastman Kodak Co., Rochester, NY), and rabbit anti-DNP IgG (RαDNP IgG) exactly as described (2).

*Preparation of Opsonized Erythrocytes.* Sheep erythrocytes (E) coated with rabbit anti-E IgG (Cordis Laboratories Inc., Miami, FL) [E(IgG)] were prepared as described (2).

*Immunoglobulins.* Control rabbit IgG was purified from pooled preimmune rabbit sera by DEAE-cellulose chromatography (12). RαDNP IgG was prepared by affinity chromatography as described (13). Monoclonal rat anti-mouse FcRII antibody 2.4G2 IgG and its Fab fragment were prepared and purified as described (8).

*Iodination of Immunoglobulins.* Immunoglobulins were iodinated using the chloramine T method of Sonoda and Schlamowitz (14). The radioiodinated RαDNP IgG ($^{125}$I-RαDNP IgG), anti-FcR 2.4G2 IgG ($^{125}$I-2.4G2 IgG), and 2.4G2 Fab ($^{125}$I-2.4G2 Fab) were indistinguishable in their biological activities from the noniodinated products.

*Binding and Phagocytosis Assays.* Binding and/or phagocytosis of E(IgG) was performed as described (2). The number of E(IgG) bound or ingested per 100 macrophages is the attachment or phagocytosis index, respectively.

*Quantitation of RαDNP IgG Binding to Coverslips.* PLL- or PLL-DNP-coated coverslips on Costar plate covers were overlayed with 0.05 ml of $^{125}$I-RαDNP IgG (2–8 × $10^6$ cpm/μg) in phosphate-buffered saline (PBS) with $Ca^{++}$ and $Mg^{++}$ and 1 mg/ml bovine serum albumin (BSA) at room temperature for 45 min. The coverslips were washed 10 times in two successive beakers containing 100 ml each of this PBS-BSA. Residual fluid was removed from the coverslips by aspiration, their uncoated bottom surface was wiped dry, and their bound radioactivity was measured in a Packard Auto-Gamma Spectrometer (Packard Instrument Co., Inc., Downers Grove, IL). At least two coverslips were used for each determination. The values obtained in the various experiments using the same lots of $^{125}$I-RαDNP IgG differed by no more than 12%.

*Quantitation of Macrophage FcR and Other Membrane Proteins on Intact Cells Using $^{125}$I-labeled Monoclonal Antibodies.* 5 × $10^5$ resident peritoneal cells or 2.5 × $10^5$ thioglycollate-elicited peritoneal cells in 0.1 ml MEM with Earle's salt solution were plated directly onto PLL- or PLL-DNP-coated coverslips, incubated for 1 h at 37°C, and washed extensively to remove nonadherent cells. At the end of an experiment, the coverslip cultures were placed into Costar wells in cold (2–3°C) Hanks' balanced salt solution (HBSS) without sodium bicarbonate and phenol red, containing 0.02 M Hepes (Sigma Chemical Co., St. Louis, MO), pH 7.2, and 1 mg/ml BSA, on ice in a cold room for 15 min. The coverslips were then removed from the wells and residual fluid was aspirated; they were then placed with their cell-free surface down onto a precooled Costar plate cover on ice, overlaid with $^{125}$I-labeled monoclonal antibody or Fab fragments in 0.05 ml of ice-cold PBS-BSA, protected with another Costar tray cover, and buried in ice. After 1 h the PBS-BSA was aspirated from the coverslips and unbound $^{125}$I-immunoglobulin was removed by dipping the coverslips 10 times into two beakers each containing 100 ml of ice-cold $Ca^{++}$- and $Mg^{++}$-free PBS (PD) and 1 mg/ml BSA. The macrophages were fixed in 2.5% glutaraldehyde in ice-cold PBS and cell-bound radioactivity was measured in a gamma counter as described above. All measurements were done in duplicate, and the average of the two determinations is expressed as ng $^{125}$I-immunoglobulin bound per coverslip. All results have been corrected for nonspecific adsorption of the same labeled immunoglobulin to cell-free coverslips that were prepared and processed in parallel with the experimental samples.

The number of macrophages on coverslips was quantitated using a calibrated eyepiece micrometer (Carl Zeiss, Inc., Thornwood, NY) to count glutaraldehyde-fixed or Wright-Giemsa-stained cells in at least 250 successive fields (3.2% of the surface area of a coverslip) at × 500 magnification. Autoradiographic localization of $^{125}$I-2.4G2 IgG was performed as described (15) using an exposure time of 24–28 h.

## Results

*Removal of Macrophage FcR by Substrate-adherent Antigen-Antibody Complexes.* Mouse peritoneal macrophages cultured on surfaces that are coated with antigen-antibody (IgG) complexes fail to bind and ingest E(IgG) (2 and Table I), but continue to attach and phagocytose complement-coated erythrocytes, latex beads, and zymosan particles (1, 2). Our previous studies showed that this inhibition is due to the loss of trypsin-resistant FcRII activity from the nonadherent segment of the macrophage plasma membrane (2). This selective inhibition of FcRII activity could be due to the functional inactivation of these receptors, or to their physical removal from the nonadherent segment of the cell's plasma membrane. To distinguish between these two possibilities we used $^{125}$I-labeled monoclonal rat anti-mouse FcRII antibody 2.4G2 and its $^{125}$I-labeled Fab fragments (9) as probes for FcRII antigen. 2.4G2 IgG and its Fab fragment specifically recognize FcRII of mouse macrophages and several other FcR-bearing cells and cell lines of mouse origin (16). The iodinated immunoglobulins were added to macrophages plated on DNP- and RαDNP IgG-coated coverslips. The incubations were performed at 2–3°C to prevent pinocytic uptake of these immunoglobulins (17). Control experiments showed that 1 h was sufficient to allow maximal binding of $^{125}$I-2.4G2 IgG to macrophages at 2–3°C and that maximal binding was achieved using 170 ng 2.4G2 IgG and 210 ng 2.4G2 Fab per coverslip culture. Therefore, in all subsequent experiments each macrophage coverslip culture was incubated for 1 h with 200 ng of monoclonal $^{125}$I-labeled IgG or 300 ng of its $^{125}$I-labeled Fab fragments.

TABLE I

*Modulation of FcRII on Resident and Thioglycollate-elicited Macrophages by Immobilized Immune Complexes as Measured by Inhibition of Binding of Monoclonal Anti-FcRII IgG and E(IgG)*

| Macrophages* | Treatment of PLL-DNP-coated coverslips | Binding of $^{125}$I-2.4G2 IgG‡ | | Binding of $^{125}$I-2.4G2 Fab‡ | | Binding of E(IgG)§ | |
|---|---|---|---|---|---|---|---|
| | | ng bound | Percent control | ng bound | Percent control | Attachment index | Percent control |
| Resident | None | 5.60 ± 0.11 | 100 | ND¶ | — | 770 ± 133 | 100 |
| | RαDNP IgG‖ | 2.21 ± 0.08 | 40 | ND | — | 37 ± 21 | 5 |
| Thioglycollate elicited | None | 5.05 ± 0.56 | 100 | 4.68 ± 0.52 | 100 | 1,281 ± 278 | 100 |
| | RαDNP IgG‖ | 1.34 ± 0.31 | 25 | 1.58 ± 0.27 | 31 | 213 ± 34 | 17 |
| | Control R IgG‖ | 4.90 ± 0.48 | 97 | ND | — | ND | — |

* Resident macrophages were cultured for 20 h and released from the monolayer with Lidocaine as described in Materials and Methods. They were plated at $2 \times 10^5$ viable macrophages per coverslip. Thioglycollate-elicited peritoneal cells were plated at $2.5 \times 10^5$ cells per coverslip. As assayed by light microscopy (see Materials and Methods) control coverslips contained $1.77 \pm 0.2 \times 10^5$ each and RαDNP IgG-coated coverslips contained $1.9 \pm 0.15 \times 10^5$ thioglycollate-elicited macrophages, respectively. The results reported are the mean ± SD of 2–6 experiments using duplicate coverslips in each experiment.

‡ Each coverslip was incubated for 1 h at 2–3°C with 0.05 ml PBS-BSA containing 200 ng $^{125}$I-2.4G2 IgG or 300 ng $^{125}$I-2.4G2 Fab. Results are corrected for background binding (0.06 ± 0.08 ng for $^{125}$I-2.4G2 IgG; 0.52 ± 0.02 ng for $^{125}$I-2.4G2 Fab) to PLL-DNP-coated coverslips without cells.

§ Binding of E(IgG) was performed for 1 h at 2–3°C by adding 0.1 ml of a 1% suspension of E(IgG) to each coverslip culture in Costar wells containing 0.5 ml ice-cold PBS as described (2). Macrophages used in this assay were treated with trypsin in suspension (2) to remove the trypsin-sensitive FcRI before plating on coverslips.

‖ RαDNP IgG and control rabbit IgG (R IgG) were used at 0.25 mg/ml in PD as described previously (2).

¶ Not done.

Resident macrophages bound an average of 5.6 ng 2.4G2 IgG per $2.0 \times 10^5$ macrophages and thioglycollate-elicited macrophages bound 5.05 ng 2.4G2 IgG and 4.68 ng 2.4G2 Fab per $1.77 \times 10^5$ macrophages (Table I). Assuming each 2.4G2 IgG binds two FcRII and each Fab binds one FcRII these data indicate that 20-h explanted resident macrophages have about $2.22 \times 10^5$ FcRII per macrophage and freshly explanted thioglycollate-elicited macrophages have between 2.26 and $3.18 \times 10^5$ (an average of $2.7 \times 10^5$) FcRII receptors per macrophage. These values agree favorably with those reported previously (8, 13) for resident and thioglycollate-elicited macrophages using immune complexes containing rabbit IgG to enumerate FcRII.

Macrophages plated on DNP-R$\alpha$DNP IgG-coated coverslips bound 25–40% as much 2.4G2 IgG or Fab as macrophages plated on control coverslips (Table I). Approximately the same concentration of immunoglobulin was required to achieve saturation of FcRII of macrophages on immune complex-coated and control substrates (data not shown), indicating that the interaction of macrophages with immune complexes does not alter the affinity of unligated Fc receptors for 2.4G2 IgG. Parallel experiments confirmed that 80% of the macrophages on DNP-R$\alpha$DNP IgG complexes did not bind or ingest E(IgG) (Table I and reference 2). Radioautographic analysis of the distribution of $^{125}$I-labeled 2.4G2 IgG on macrophages plated on immune complex-coated substrates showed reduced binding of the immunoglobulin by nearly all the macrophages in the culture (data not shown). Thus the observed reduction in 2.4G2 IgG binding reflects an effect of the substrate-adherent immune complexes on all of the cells in the culture, and not on a selected subpopulation.

These results demonstrate that macrophages respond to the presence of immobilized Fc ligands with a reduction in FcRII antigen on their surface membranes. Thus inhibition of FcR activity by immobilized antigen-antibody complexes is a direct result of the removal of Fc receptors from the nonadherent surface of the macrophage plasma membrane.

Experiments reported in a companion paper (9) confirm the selectivity of FcRII removal. They show that macrophages plated on DNP R$\alpha$DNP IgG-coated surfaces exhibit a much greater reduction in 2.4G2 IgG binding than in the binding of another monoclonal antibody directed against a macrophage surface antigen unrelated to FcRII.

*Effect of Varying Ligand Concentration of FcR Modulation.* To determine the number of substrate-adherent ligands required to promote FcR modulation we bound varying amounts of $^{125}$I-labeled R$\alpha$DNP IgG to PLL- and PLL-DNP-coated coverslips. Using the specific activity of the radiolabeled R$\alpha$DNP IgG we calculated the amount of R$\alpha$DNP IgG bound per coverslip (Fig. 1). As expected, DNP-coated coverslips bound much more R$\alpha$DNP IgG at low immunoglobulin concentrations than coverslips coated with PLL alone. Measurable adsorption of R$\alpha$DNP IgG to coverslips coated with PLL alone required the addition of at least a 10-fold higher antibody concentration than to PLL-DNP-coated coverslips. At the highest concentrations of R$\alpha$DNP IgG used, PLL-coated coverslips bound about one third as much R$\alpha$DNP IgG as PLL-DNP-coated coverslips. It should be emphasized that the antibody was added to only one surface of the coverslip and therefore all IgG were bound only to that surface.

Macrophage FcR function was extremely sensitive to low concentrations of R$\alpha$DNP IgG (0.01–1 $\mu$g), but only if macrophages were plated on the coverslips coated with PLL-DNP. FcR function was only slightly affected, even at the highest R$\alpha$DNP

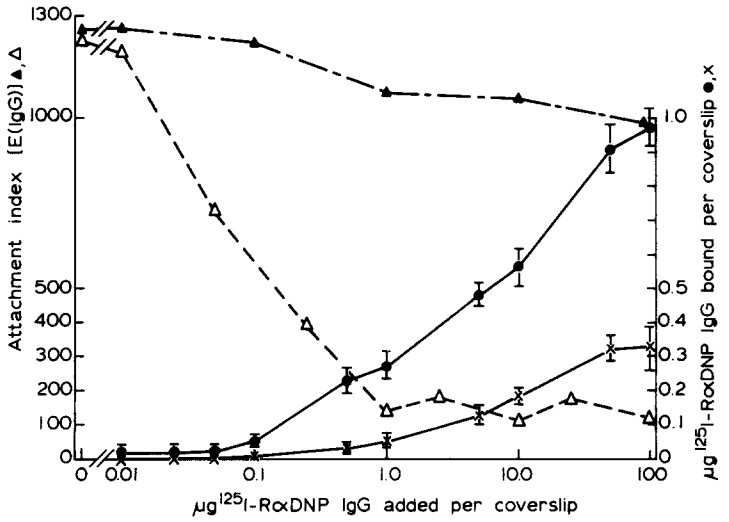


FIG. 1. Quantitation of the amount of RαDNP IgG bound to PLL- and to PLL-DNP-coated coverslips. $^{125}$I-RαDNP IgG in the amount indicated on the abscissa was incubated at room temperature with glass coverslips coated with PLL (×) or with PLL-DNP (●) under conditions that allowed binding of radiolabeled IgG only to the coverslips' PLL- or PLL-DNP-coated surface. The coverslips were washed and assayed for $^{125}$I-RαDNP IgG. The remaining coverslips were overlaid with $1.5 \times 10^5$ thioglycollate-elicited peritoneal cells for 1 h at 37°C, and then washed. The macrophages on PLL (▲)- and PLL-DNP (△)-coated coverslips were assayed at 2–3°C for E(IgG) binding. Each data point is the average of duplicate coverslips.

concentration used (100 μg), when the macrophages were plated on PLL-coated coverslips (Fig. 1). At this concentration the PLL-coated coverslips adsorbed 0.33 μg RαDNP IgG, an amount of IgG sufficient to cause maximal FcR modulation in macrophages on PLL-DNP-coated coverslips. The failure of IgG adsorbed to PLL-coated coverslips to modulate Fc receptors suggests that FcRII has a much higher affinity for IgG-antigen complexes than for IgG uncomplexed with antigen.

A more complete analysis of the relationship between the amounts of RαDNP IgG complexed to DNP-coated coverslips and its effect on macrophage Fc receptor function is shown in Figs. 2 and 3. Inhibition of E(IgG) binding and ingestion was first observed when $1 \times 10^{10}$ molecules of RαDNP IgG were bound to each coverslip. This is an estimated value since binding of this amount of $^{125}$I-RαDNP IgG could not be detected above background (Figs. 1 and 2). Binding of RαDNP IgG to DNP-coated coverslips could be accurately assessed when 0.01 μg of $^{125}$I-RαDNP IgG was used. At this level ($4 \times 10^{10}$ molecules) virtually all of the radiolabeled IgG added was bound to the coverslips and caused ~15% reduction in E(IgG) binding and ingestion. However, we observed no reduction in binding of $^{125}$I-2.4G2 IgG until $6 \times 10^{10}$ molecules of RαDNP IgG were bound to the substrate. At higher RαDNP IgG concentrations, roughly the same amount of substrate-bound IgG (0.3 μg, $1.2 \times 10^{12}$ molecules) was needed to produce maximal inhibition of E(IgG) and 2.4G2 IgG binding and of E(IgG) phagocytosis.

At least two factors could account for the apparent differences in amounts of substrate-adherent RαDNP IgG required for inhibition of E(IgG) binding vs. $^{125}$I-2.4G2 IgG binding. First, to facilitate enumeration of E(IgG) binding, fewer macrophages were plated on the coverslips used for this assay than for $^{125}$I-2.4G2 IgG

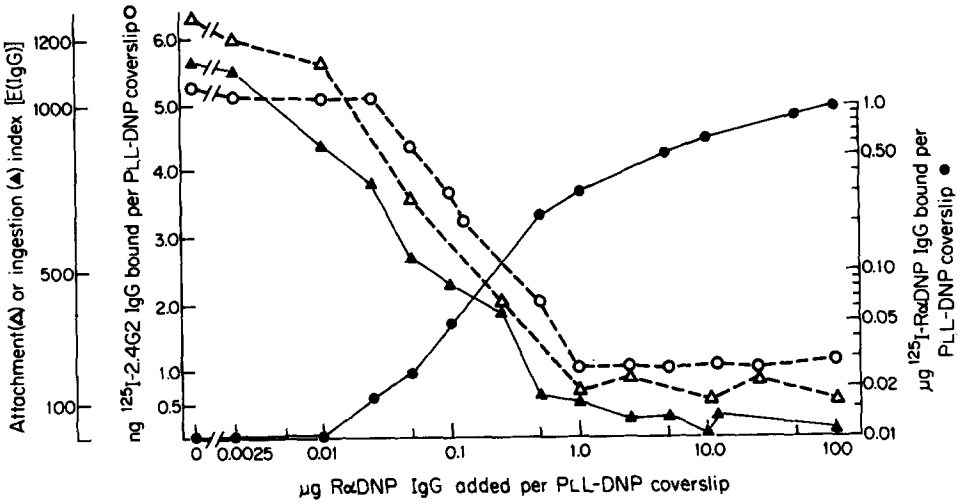


FIG. 2. Relationship between the amount of RαDNP IgG bound as immune complexes to PLL-DNP-coated coverslips and the extent of macrophage FcR modulation. PLL-DNP coverslips coated with known amounts of RαDNP IgG (as described in Fig. 3) were overlaid with thioglycollate-elicited peritoneal cells at 37°C for 1 h and washed. $1.5 \times 10^5$ peritoneal cells were plated on coverslips used for E(IgG) binding and phagocytosis; $2.5 \times 10^5$ cells were plated on coverslips used for $^{125}$I-2.4G2 IgG binding. (△) E(IgG) attachment index; (▲) E(IgG) ingestion index; (○) ng $^{125}$I-2.4G2 IgG bound; (●) μg $^{125}$I-RαDNP IgG bound by PLL-DNP-coated coverslips. Each data point is the average of two coverslips.

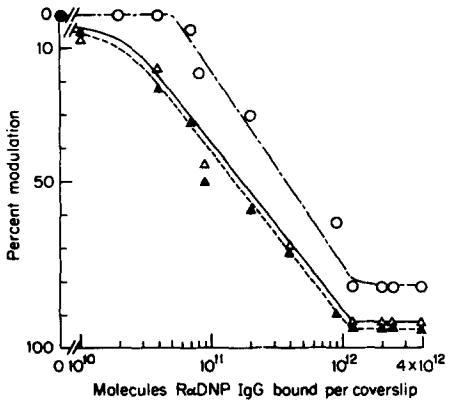


FIG. 3. Relationship between the number of molecules of RαDNP IgG bound to the substrate and the extent of FcR modulation. All data are from Figs. 3 and 4. The abscissa indicates the total number of RαDNP IgG molecules bound to PLL-DNP-coated coverslips; these values are not corrected for "nonspecific" binding of RαDNP IgG to PLL-coated coverslips. At the amount of RαDNP IgG needed to cause maximal FcR modulation (1.0 μg), nonspecific IgG binding to PLL-coated coverslips is <10% of specific binding to PLL-DNP-coated coverslips (Fig. 3). 0.01 μg RαDNP IgG contains $4 \times 10^{10}$ molecules. Attachment (△) and ingestion (▲) of E(IgG) and binding of $^{125}$I-2.4G2 IgG (○) are reported as percent of control using as 100% the values for macrophages on PLL-DNP-coated coverslips to which no RαDNP IgG was added.

binding. Second, 2.4G2 IgG may have access to FcR at sites that are inaccessible to a large particle such as E(IgG). This is especially significant at low concentrations of substrate-adherent RαDNP IgG. Under these conditions the space between the macrophage and the substrate may remain patent to 2.4G2 IgG. At higher concen-

trations of substrate-adherent immune complexes, 2.4G2 IgG is excluded from this space and thus is prevented from binding to FcR on the macrophage's undersurface (see Discussion in reference 9).

Despite these uncertainties two major conclusions emerge from these measurements. First, above the critical number of RαDNP IgG required to initiate FcR modulation (1–6 × $10^{10}$ IgG molecules bound per coverslip) there is a stoichiometric relationship between the number of IgG ligands on the substrate and the extent of FcRII modulation (Figs. 2 and 3). Second, modulation of receptors on living cells by substrate-adherent ligands occurs within a narrow concentration range, one that is roughly equivalent to the range of concentrations used in a conventional immunoassay.

*Effects on FcR of Extended Cultivation of Macrophages on Antigen-Antibody-coated Surfaces.* As indicated in Fig. 4, A and B, macrophages cultured on control substrates for up to 72 h exhibit an approximately threefold increase in their capacity to ingest E(IgG), and a 4.5-fold increase in FcRII (from 2.7 × $10^5$ to 1.08 × $10^6$ per cell), as measured by binding of $^{125}$I-2.4G2 IgG. Since macrophages are nondividing cells the

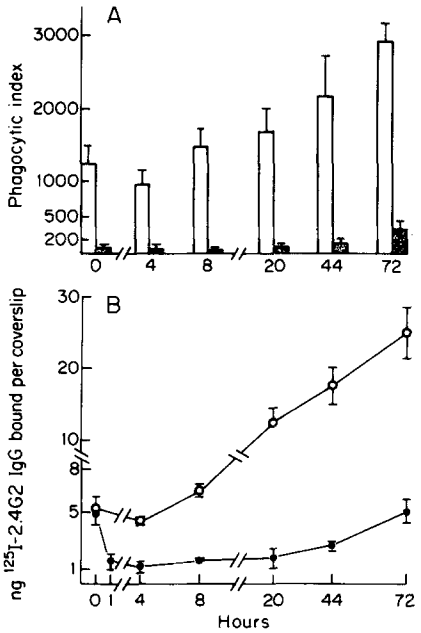


FIG. 4. Effects of long-term culture of macrophages on immune complex-coated coverslips on FcR-mediated ingestion of E(IgG) and binding of $^{125}$I-2.4G2 IgG. Thioglycollate-elicited macrophages on PLL-DNP-coated coverslips were incubated at 2°C with RαDNP IgG as described in the "sequential" method of immune complex formation (9). Control cultures were incubated at 2–3°C with PD. The cultures were washed and incubated at 37°C in MEM with Earle's salt solution for 1 h. Duplicate coverslip cultures were assayed at this time for E(IgG) ingestion or $^{125}$I-2.4G2 IgG binding as described in Materials and Methods. The remaining cultures were washed twice in warm PBS and incubated at 37° in MEM plus 10% hFBS. At the time points indicated duplicate coverslip cultures were removed and used for each assay. The data reported are the averages ± SD of three experiments. (A) 1.5 × $10^5$ peritoneal cells were plated onto each coverslip. Phagocytosis of E(IgG) by macrophages on PLL-DNP-coated (open bars) or PLL-DNP-RαDNP IgG-coated (hatched bars) coverslips. (B) 2 × $10^5$ peritoneal cells were plated onto each coverslip. Nanograms of $^{125}$I-2.4G2 IgG bound by macrophages on PLL-DNP-coated (○) or PLL-DNP RαDNP IgG (●)-coated coverslips.

increase in Fc receptor number and activity represents an absolute increase in receptor number and activity per cell.

Macrophages on DNP-RαDNP IgG-coated coverslips showed ~90% inhibition of E(IgG) ingestion and ~70% decrease in binding of $^{125}$I-2.4G2 IgG within 1 h of plating (Fig. 4, A and B). This inhibitory effect was maintained for 44 h. A small, but measurable, increase in expression of FcRII and the capacity to ingest E(IgG) occurred between 44 and 72 h of culture on these antigen-antibody-coated coverslips.

## Discussion

Decreased FcR activity of macrophages maintained on immune complex-coated surfaces is caused by the physical removal of these receptors from the surface of the cells (Table I), and occurs only when the surface-adherent IgG is complexed to antigen (Fig. 1). IgG molecules adsorbed to the substrate in the absence of antigen are ineffective in promoting FcR modulation (Fig. 1).

We have analyzed the relationship between ligand number and FcR modulation by culturing macrophages on substrates coated with a measured amount of immune complexes. This analysis was facilitated by the enormous spreading of the plasma membranes of macrophages on DNP-RαDNP IgG-coated coverslips. When plated at a concentration of $2.5 \times 10^5$ thioglycollate-elicited peritoneal cells per DNP-RαDNP IgG-coated coverslip, as was done in all experiments in which binding of $^{125}$I-2.4G2 IgG was assayed, the macrophages spread until they covered virtually the entire surface of the coverslip. For this reason we are confident that most of the RαDNP IgG bound to each coverslip lies beneath a macrophage and is in a position to ligate FcR. This visual estimate is supported by quantitative measurements of the surface area of well-spread macrophages.[2]

The inverse relationship observed between the number of IgG molecules attached to the substrate and the extent of FcR modulation (Fig. 3) does not, by itself, indicate whether the receptors are responding to the number or to the concentration of IgG ligands on the substrate. To clarify this issue we have calculated the concentration of these ligands on the substrate and have compared these values with the affinity of FcRII for soluble immune complexes.

$1.7–3.6 \times 10^{11}$ substrate-adherent IgG molecules (average, $2.65 \times 10^{11}$) are needed to promote half-maximal FcRII modulation (Fig. 3). The concentration of this number of molecules in a volume 200 Å deep[3] and covering the entire surface of a 12-mm coverslip ($1.13 \times 10^8$ $\mu m^2$) is $1.95 \times 10^{-4}$ M. In contrast, the affinity of FcRII for soluble immune complexes containing RαDNP IgG has been estimated to range from $1.4 \times 10^{-7}$ to $2.4 \times 10^{-8}$ M (13, 20). Thus the concentration of substrate-adherent IgG needed to produce 50% FcR modulation is, at a minimum, 1,000-fold greater than that needed to promote binding of immune complexes to FcR. These calculations indicate that it is the number, and not the concentration of surface-bound IgG that

[2] Each thioglycollate-elicited macrophage on a glass coverslip occupies 450 $\mu m^2$ of the coverslip's surface and has at least 900 $\mu m^2$ of plasma membrane (18). When fully spread, as occurs on DNP-RαDNP IgG-coated coverslips, each of these macrophages occupies about 1,200 $\mu m^2$ of the coverslip's surface and has over 2,400 $\mu m^2$ of plasma membrane (18). Thus the macrophages have more than enough plasma membrane [(1,200 $\mu m^2$/macrophage) × ($1.9 \times 10^5$ macrophages) = ($2.25 \times 10^8$ $\mu m^2$)] to cover the area ($1.13 \times 10^8$ $\mu m^2$) of a 12-mm diam coverslip.

[3] An IgG molecule is ~142 × 125 Å (19); 200 Å provides sufficient depth to accommodate IgG arranged perpendicularly to the plane of the coverslip and is about twice the distance between the macrophage membrane and the surface of an IgG-coated erythrocyte bound to it.

is the limiting factor in regulating FcR modulation.

Although we have measured the absolute number of RαDNP IgG molecules bound to the substrate, we do not know whether every molecule is appropriately oriented to act as a ligand. We believe that FcRII, like the FcR for IgE (21, 22), is monovalent. If so, the substrate must bear at least one functional ligand (i.e., in the appropriate orientation to bind an FcR) for each receptor modulated. Thus it is possible that of the $1.2 \times 10^{12}$ molecules needed to produce maximal receptor modulation only 5% ($6 \times 10^{10}$) are appropriately oriented to act as ligands. If this occurs then our estimate of the number of ligands needed to initiate FcR modulation would be correspondingly reduced to $3 \times 10^9$ IgG molecules per coverslip, a value that is <10% of the total number of FcR in the system. The concentration of this number of ligands in a volume 200 Å deep and covering the coverslip's surface is $2.2 \times 10^{-6}$ M, a value that is still 15–100-fold in excess of the Ka of FcRII.

Regardless of the exact ratio between FcRII and the number of functional Fc domains on the coverslip, there is good agreement between the number of substrate-adherent IgG molecules required to achieve half-maximal inhibition of uptake of E(IgG) ($1.7 \times 10^{11}$ molecules) and half-maximal inhibition of $^{125}$I-2.4G2 binding ($3.6 \times 10^{11}$ molecules). Maximal inhibition was achieved at the same level of RαDNP IgG ($1.2 \times 10^{12}$ molecules) in both assays. The potential availability of an intracellular pool of FcR makes it difficult to interpret our finding that the number of substrate-adherent ligands needed to initiate FcR modulation ($6 \times 10^{10}$) is approximately equal to the total number of FcRII molecules ($5.13 \times 10^{10}$) on all the macrophages on a coverslip. FcR ligation might promote transfer of receptors from intracellular membranes to the plasma membrane, thereby replenishing surface FcR. (In the case of glucose transporters in fat cells (23) and mannose-6-phosphate receptors in fibroblasts (24), the number of intracellular receptors is several-fold the number of receptors on the cell's surface.) If such transfer occurred in our experiments the number of substrate-adherent ligands needed to cause a measurable decrease in surface FcR would be dependent upon the size of the intracellular pool of receptors and larger than predicted from measurements of the number of surface receptors. Indeed, this is consistent with our observations (Fig. 4).

Insertion of newly synthesized receptors also could explain the reappearance of FcR activity in macrophages cultured for 72 h on immune complexes (Fig. 4). Complete FcR modulation requires a 20-fold excess of substrate-adherent ligands over membrane receptors (Figs. 2 and 3). The coverslips used in the experiment described in Fig. 4 bore $2.4 \times 10^{12}$ RαDNP IgG molecules. Assuming that FcRII synthesis and insertion continues at the same rates in macrophages plated on control and on immune complex-coated coverslips, macrophages cultured for 72 h on these complexes should bear $1.08 \times 10^6$ FcRII each (open circles, Fig. 4), a number of FcR sufficient to reduce the ratio of substrate-adherent ligands to FcRII to about 12 [$2.4 \times 10^{12}$ RαDNP IgG/($1.08 \times 10^6$ FcRII) × ($1.9 \times 10^5$ macrophages)]. At this ligand/receptor ratio we observed incomplete modulation of FcR (~20–35% of control) in freshly explanted macrophages (Fig. 3), and therefore might expect to observe a similar level of FcR activity in macrophages cultured for 72 h on immune complexes. This was in fact the case as is shown in Fig. 4.

Further work is needed to define the relationship between the concentrations of surface-bound IgG needed to promote modulation of FcR and the binding and

ingestion of opsonized erythrocytes by macrophages. Nevertheless, it is important to emphasize the utility of the experimental approach used here. The physical displacement of FcR from the apical to the substrate-adherent membrane segment converts the macrophages from randomly oriented to polarized cells. This polarization may have important functional consequences such as the vectorial release of granules in polymorphonuclear leukocytes (25) and mast cells (26). Ligand-coated surfaces may also induce vectorial secretion of reactive oxygen metabolites, bioactive lipids, or neutral proteases by macrophages, thereby producing extremely high local concentrations of these effector molecules (27). The system described here is ideally suited to probing such questions.

## Summary

Macrophages plated on surfaces coated with antigen-IgG complexes lose the capacity to bind and ingest IgG-coated particles via their Fc receptors (FcR). Macrophages plated on surfaces containing a similar number of IgG molecules that are not complexed to antigen show little or no decrease in FcR activity. Using a rat monoclonal antibody (2.4G2 IgG) directed against the trypsin-resistant FcR (FcRII) of mouse macrophages we show that the decrease in receptor activity induced by substrate-adherent immune complexes is caused by the physical removal of 60 and 75% of FcRII from the nonadherent membrane surfaces of resident and thioglycollate broth-induced macrophages, respectively. Macrophages maintained on antigen-IgG-coated surfaces for up to 44 h show no recovery in FcRII activity or number, while macrophages on control surfaces exhibit two and threefold increases, respectively, in these parameters. Macrophages maintained for 72 h on antigen-IgG-coated surfaces show a small recovery in FcRII activity, and in the number of FcRII that is accessible to bind $^{125}$I-2.4G2 IgG.

FcRII modulation, as measured by the binding of $^{125}$I-labeled 2.4G2 IgG, is initiated when the number of IgG molecules bound to the substrate is approximately equal to the total number of FcRII on the plasma membranes of all the macrophages on the substrate. FcRII activity and number decrease linearly as the number of substrate-bound IgG molecules increases exponentially, and are maximally reduced when the number of IgG molecules on the substrate is 20-fold greater than the total number of all FcRII on the surfaces of all the macrophages in the culture. Thus there is a stoichiometric relationship between the number of IgG molecules on the substrate and the extent of FcRII modulation.

*Received for publication 22 November 1982 and in revised form 7 February 1983.*

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