Internalization and Degradation of Macrophage Fc Receptors Bound to Polyvalent Immune Complexes

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ABSTRACT We have studied the Fc receptor-mediated pinocytosis of immunoglobulin G (IgG)-containing immune complexes by mouse macrophages. IgG complexes were formed from affinity-purified rabbit dinitrophenyl IgG and dinitrophenyl modified BSA at molar ratios of 2.5–10:1. Both the specificity of binding and the fate of internalized receptors were analyzed using monoclonal and polyclonal anti-Fc receptor antibodies. Based on the susceptibility of surface-bound ligand to release by proteolysis, we have found that at 37°C, ^{125}I-labeled IgG complexes were rapidly internalized ($t_{1/2}$ <2 min) and delivered to lysosomes; acid-soluble ^{125}I was detectable in the growth medium within 5–10 min of uptake. However, kinetic evidence indicated that Fc receptors were not efficiently re-used for multiple rounds of ligand uptake. Instead, macrophages that were exposed continuously to saturating concentrations of IgG complexes exhibited a selective and largely irreversible removal of Fc receptors from the plasma membrane. This loss of surface receptors correlated with an increased rate of receptor turnover, determined by immune precipitation of Fc receptors from ^{125}I-labeled macrophages. Thus, in contrast to the results obtained in the accompanying paper (I. Mellman, H. Plutner, and P. Ukkonen, 1984, *J. Cell Biol.* 98:1163–1169) using a monovalent ligand, these data indicate that the interaction of Fc receptors with polyvalent complexes leads to the degradation of both ligand and receptor following their delivery to lysosomes.

In macrophages, Fc receptors (FcR)[1] along with many other plasma membrane proteins are continuously internalized and recycled during endocytosis (1, 2). Several considerations have led to this conclusion. For example, evidence presented in the accompanying paper (3) indicates that the monovalent Fab fragment of an antireceptor antibody can be internalized and then rapidly returned to the cell surface still bound to FcR. Although the precise intracellular pathway taken by these Fab-FcR complexes is not entirely clear, recycling appears to occur primarily from a low density prelysosomal endocytic compartment, i.e., endosomes (4).

On the other hand, the intracellular pathway and fate of FcR bound to multivalent ligands may be quite different. In previous work (5), we have shown that the phagocytosis of large, immunoglobulin G (IgG)-coated particles effectively removes FcR from the recycling pathway, with receptors being rapidly degraded following the fusion of incoming phagocytic vacuoles with macrophage lysosomes. Since phagocytosis may by-pass a compartment such as the endosome from which receptor recycling must occur, we have now examined the internalization and fate of FcR during the pinocytosis of soluble IgG-containing immune complexes (6–8). Recent electron microscopic study has shown that IgG-complexes are rapidly taken up by macrophages by coated pits-coated vesicles and delivered to endosomes prior to their appearance in electron-dense lysosomes (2, 9). In spite of this endosome intermediate, we have found that the receptor (along with its bound ligand) is still transferred to and degraded in lysosomes. Taken together with the results of the accompanying paper (3), these data suggest that ligand-induced clustering of adjacent FcR prevents recycling and results in the routing of internalized receptors in lysosomes.

[1] *Abbreviations used in this paper:* DNP, dinitrophenyl; FcR, Fc receptors; α8-H, α-modified Eagle's medium containing 8% fetal calf serum and 10 mM Hepes.

MATERIALS AND METHODS

Cell Culture: The mouse macrophage cell line J774 was maintained in suspension culture as described (3). For most experiments, cells were plated 60 min before use in 24-well tissue culture plates (3–4 × 10^5 cells/16-mm well) in α-modified Eagle's medium containing 8% fetal calf serum and 10 mM HEPES (pH 7.2) (α8-H). Thioglycollate-elicited peritoneal macrophages were obtained from CD_2F_1 (BALB/c × DBA/2) mice as described (10) and grown in α8-H. Monolayers were rinsed several times with cold PBS containing 5 mM glucose (PBS-G) prior to use.

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Antibodies and Immune Complexes: Rabbit antidinitrophenyl (DNP) IgG was prepared by affinity chromatography as described (11). Purified antibody was stored at 4°C in PBS containing 0.02% NaN_3. Immune complexes were formed just before use by combining (37°C, 30 min) anti-DNP IgG (20 μg/ml) and DNP-modified bovine serum albumin (DNP·BSA) at molar ratios of 2.5:1 to 10:1 (usually, 2.5:1). ^{125}I-IgG was not sedimented by centrifugation at 100,000 *g* for 1 h, indicating that large, insoluble aggregates were not formed under these conditions. IgG complexes chromatographed on Sepharose 4B as a broad peak with an average molecular weight of 4–5 × 10^5, as described previously (6). DNP·BSA was prepared as described (12) using dinitrobenzene sulfonic acid (Eastman Kodak, Rochester, NY) to yield 17–35 mol of DNP/mol of BSA.

Monoclonal rat anti-mouse macrophage antibodies were produced and purified as described previously (13). The monovalent Fab fragment of the anti-FcR monoclonal antibody 2.4G2 was prepared using papain (Sigma Chemical Co., St. Louis, MO) and purified by chromatography on DEAE-cellulose (14). All antibody preparations used were homogeneously pure as judged by SDS PAGE.

Iodinations: Proteins were labeled at 4°C with ^{125}I using Iodogen (Pierce Chemical Co., Chicago, IL) (15) and separated from unincorporated radiolabel by chromatography on a 0.4-ml column of Dowex 1-X8 (200–400 mesh) (5). 0.5–1.0 mCi Na^{125}I (carrier-free; Amersham/Searle, Arlington Heights, IL) was added per iodination (50 μg protein in 0.1 ml PBS); 50–80% incorporation of ^{125}I into protein was routinely obtained.

J774 cells were iodinated at 4°C in suspension (3–4 × 10^7 cells/ml, 2 mCi Na ^{125}I/ml) using a slight modification (13) of the lactoperoxidase-glucose oxidase technique of Hubbard and Cohn (16). Cell viability (>96% by trypan blue exclusion) was not affected by this procedure. Following labeling, cells were washed in cold serum-free medium, plated in 35-mm dishes (2 × 10^6 cells/dish) in α8-H, and allowed to attach for 30 min at 37°C before use. Iodination did not affect the ability of surface FcR to bind IgG complexes.

Binding and Internalization Assays: ^{125}I-labeled IgG-DNP·BSA complexes were formed in α8-H and then added (0.2 ml) to cell monolayers in 16-mm wells (3–4 × 10^5 cells/well). Following incubation at 4° or 37°C, cells were washed five times with cold PBS-G and harvested using a cotton-tipped swab. ^{125}I was then determined in a Beckman (Beckman Instruments, Inc., Palo Alto, CA) gamma scintillation spectrometer. Nonspecific binding was routinely measured in parallel wells to which 100 μg/ml of the anti-FcR monoclonal antibody 2.4G2 IgG had been added (17). At 4°C, 2.4G2 IgG inhibits the binding of saturating concentrations of ^{125}I-IgG-DNP·BSA by ~90% (see Results). A portion of the uninhibitable binding may be due to the attachment of ^{125}I-complexes to a second FcR (specific for monomeric IgG2a) which is not recognized by 2.4G2 (14, 17). On J774 cells, the number of these IgG2a FcR is some 10-fold less than the number of immune complex-specific FcR (8 × 10^5/cell) which are recognized by 2.4G2 (unpublished results).

Surface-bound and intracellular ^{125}I-IgG-DNP·BSA were distinguished by the susceptibility of the former to removal by protease treatment at 4°C. Following incubation at 4° or 37°C in ^{125}I-IgG complexes, washed monolayers were incubated for 2 h at 4°C in 0.5 ml PBS containing 1 mg/ml subtilisin BPN (nagarase; Sigma Chemical Co., St. Louis, MO), 10 mM dithiothreitol, and 5 mM EDTA. This treatment routinely removed 85–90% of the total ^{125}I from cells incubated with ^{125}I-IgG-DNP·BSA at 4°C, under which conditions 100% of the cell associated radiolabel was assumed to be on the cell surface. Subtilisin treatment did not, however, inactivate the activity or antigenicity of the FcR. Of the many proteases tested, only bromelain (1 mg/ml; Sigma Chemical Co.) proved as effective as subtilisin. PBS containing 10 mM dithiothreitol and 5 mM EDTA was only slightly more effective than PBS alone (<25% removed). Incubation with 100 μg/ml 2.4G2 IgG for 2 h at 4°C was only ~70% efficient. Low pH (2.5–5.5) was ineffective at removing surface bound complexes at 4° or 37°C.

Monoclonal Antibody Binding Assay: The binding of ^{125}I-labeled monoclonal antimacrophage antibodies was performed at 4°C using saturating concentrations of antibody (1–2 μg/ml) as described (5). Nonspecific binding was determined by including a large excess (200 μg/ml) of unlabeled antibody during the incubation. Similarly, the binding of these antibodies via their Fc domains to plasma membrane FcR (as opposed to their respective membrane protein antigens) was assessed by including 100 μg/ml 2.4G2 IgG in the incubation mixture in some experiments.

Fluorescence Microscopy: Cells were placed on round glass coverslips and incubated in unlabeled IgG-DNP BSA at 4° or 37°C. Following washing in cold PBS-G, monolayers were fixed at room temperature (10 min) with 3% paraformaldehyde in PBS, rinsed with PBS, and incubated in 50 mM NH_4Cl in PBS (10 min) to quench any remaining aldehyde groups. For some experiments, cells were then permeabilized with 0.1% Triton X-100 in PBS (4 min). Rabbit immune complexes were visualized using rhodamine conjugated F(ab')$_2$ fragments of affinity-purified goat anti-rabbit IgG (Tago, Inc., Burlingame, CA) (diluted 1:40 in PBS containing 0.2% gelatin). Coverslips were mounted in Moviol (the generous gift of Daniel Louvard, Pasteur Institute) and examined using a Zeiss photomicroscope equipped with an epifluorescence illuminator.

Immune Precipitation: Precipitation of ^{125}I-labeled plasma membrane antigens from iodinated J774 cells was performed as described previously (13). ^{125}I-FcR was immunoprecipitated using a rabbit antireceptor antiserum, raised against purified J774 FcR, which recognizes FcR both in the presence and absence of bound IgG-DNP·BSA complexes (5). Precipitated proteins were displayed on 4–11% SDS polyacrylamide gradient gels (18).

RESULTS

Binding and Internalization of IgG-DNP·BSA

At 4°C, the binding of IgG-DNP·BSA complexes to J774 cells saturates within 2 h and at IgG concentrations of 20 μg/ml. That this binding was largely specific for FcR was demonstrated by its sensitivity to inhibition by the anti-FcR monoclonal antibody 2.4G2. In Fig. 1, "2.4G2-specific" binding was determined by subtracting the amount of ^{125}I-IgG that was bound in the presence of 100 μg/ml 2.4G2 IgG from the "total" amount of ^{125}I-IgG bound in the absence of 2.4G2. In all subsequent experiments, only "2.4G2-specific" binding is given, which usually represented between 80–90% of the total IgG bound at 20 μg/ml. At saturation, ~1 × 10^6 molecules of IgG were bound per J774 cell, a value in agreement with the number of surface FcR (8 × 10^5/cell) determined from the binding of ^{125}I-2.4G2 Fab (see reference 17 and Table I, below). The apparent K_A, determined from double reciprocal plots of the data in Fig. 1, was ~2 × 10^7 M^{-1}, as previously reported (6, 11, 17). Qualitatively similar results were obtained with complexes formed at IgG-BSA ratios of 2.5–10:1.

As shown in Fig. 2, ^{125}I-IgG complexes bound at 4°C were rapidly internalized upon warming the cells to 37°C. The rate of internalization, estimated from the rate at which cell-

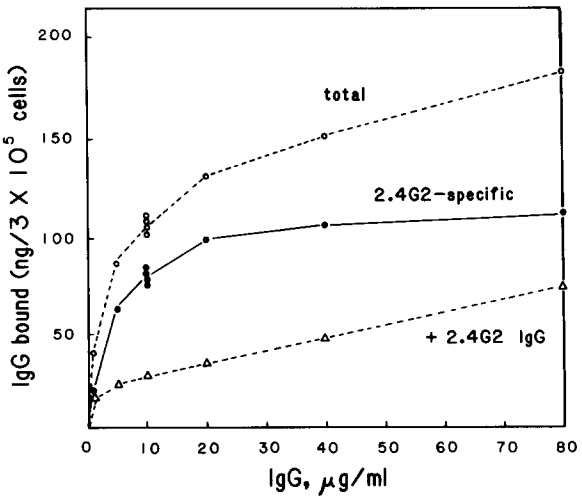

FIGURE 1 Binding of ^{125}I-IgG-DNP·BSA immune complexes to J774 cells. 3 × 10^5 J774 cells were plated in 16-mm wells and incubated for 2 h at 4°C in α8-H containing IgG-complexes at the indicated concentrations (abscissa). Identical results were obtained using 10:1 complexes formed at IgG concentrations of 20, 40, or 80 μg/ml (e.g., multiple points at 10 μg/ml). The fraction of the total cell associated ^{125}I bound to FcR was estimated by including the antireceptor monoclonal antibody 2.4G2 (100 μg/ml), which competes with IgG complexes for binding to FcR (14, 17). Specific binding (●) was determined by subtracting the amount of ^{125}I-IgG-complexes bound in the presence of 2.4G2 (△) from the total amount of cell associated radiolabel (○) bound in the absence of 2.4G2.

TABLE I

Selective Removal of Fc Receptors from the Plasma Membrane of J774 Cells During IgG-Immune Complex Pinocytosis

	Antigen (sites/cell)*				
	Fc Receptor	1.21J	2D2C	H-2D^d	2E2A
Control cells	7.12	1.14	2.15	3.12	0.91
IgG complexes‡	2.52	1.26	2.20	3.10	0.90
Percent change	−65%	+10%	+2%	<1%	<1%

All values (other than the percentages) are $\times 10^{-5}$.

* Fc receptor number determined using ^{125}I-labeled Fab fragment of 2.4G2. All other antigens quantitated using intact ^{125}I-IgG's. Binding was for 1 h at 4°C using antibody concentrations of 1 μg/ml (except for the monoclonal anti-H-2D^d antibody, B25-1, which was used at 5 μg/ml). 1.21J recognizes two polypeptides 180 and 94 kdaltons; 2D2C recognizes a 90 kdaltons glycoprotein; 2E2A recognizes a 84 kdaltons glycoprotein (13).

‡ 2 h at 37°C.

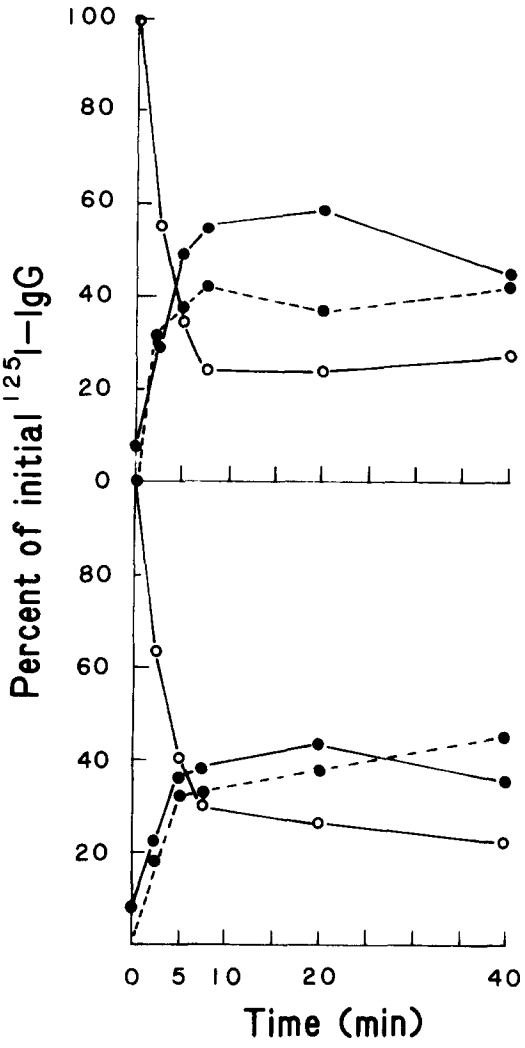

FIGURE 2 Internalization of FcR-bound ^{125}I-IgG-DNP·BSA immune complexes. ^{125}I-IgG-complexes were bound to J774 cells at 4°C (2 h) at 20 μg/ml (*top*) or 1 μg/ml (*bottom*). Cultures were rapidly warmed to 37°C in a gently agitating water bath and at various times thereafter, cooled and treated with subtilisin to quantitate cell surface bound (subtilisin-releasable) (○) and intracellular (subtilisin-resistant) (●) ^{125}I. Radiolabel lost to the growth medium (●- - -●) was also determined and found to be largely TCA-precipitable.

associated radiolabel became resistant to removal by subtilisin (see Materials and Methods), exhibited an apparent $t_{1/2}$ of 1.5–2 min. Identical rates were observed whether complexes were bound at saturating (20 μg/ml) or subsaturating (1 μg/ml) conditions (Fig. 2). The absence of a concentration dependence suggested that internalization was not preceded by the formation of higher order aggregates of surface-bound complexes.

As observed for other ligands, such as low density lipoprotein (19), a substantial fraction (40–55%) of the radiolabel that had been bound at 4°C rapidly appeared in the medium upon warming. Initially, all of this ^{125}I was TCA-precipitable, suggesting dissociation of ^{125}I-IgG from the surface. After 15–30 min, however, small amounts of TCA-soluble radioactivity were detected, suggesting the degradation of ^{125}I-IgG-DNP·BSA in lysosomes. However, degradation of internalized ligand was more effectively studied using complexes formed labeled DNP·BSA, the BSA portion of the complex being digested several times more rapidly than the IgG portion (see below). When IgG-^{125}I-DNP·BSA complexes were bound to J774 cells at 4°C, TCA soluble ^{125}I was detected in the medium within 5–10 min after warming (Fig. 3). Thus, multivalent IgG-DNP·BSA complexes bind to FcR at 4°C and upon warming, are rapidly internalized and degraded, presumably in lysosomes.

Steady-state Internalization of IgG Complexes

To determine whether FcR are reutilized for multiple rounds of ligand uptake, we next studied internalization by cells continuously exposed to IgG complexes at 37°C. The localization of cell-associated complexes was first examined by indirect immunofluorescence using rhodamine-labeled goat anti-rabbit IgG. After a 2-h incubation at 4°C with 20 μg/ml IgG-DNP·BSA, complexes were seen on the plasma membrane of J774 cells (Fig. 4, *A* and *B*). In contrast, after 2 h at 37°C, relatively little cell surface-bound ligand was detected (Fig. 4, *C* and *D*). Since saturating concentrations of IgG-complexes were present continuously in the growth medium, the lack of surface-bound ligand suggested the loss of surface FcR. Significant amounts of ligand had been internalized, however, as indicated by the staining of cytoplasmic vacuoles in cells permeabilized with Triton X-100 prior to the addition of the fluorescent second antibody (Fig. 4, *E* and *F*).

Ligand uptake was also studied quantitatively using ^{125}I-labeled IgG-complexes; surface and intracellular radiolabel were distinguished on the basis of sensitivity to removal by subtilisin treatment in the cold (see Materials and Methods). As shown in Fig. 5, the total amount of cell-associated ^{125}I-IgG-complexes increased rapidly, reaching a plateau within 30 min, which was maintained for several hours. At early times, most of this radiolabel was surface-bound (i.e., subtili-

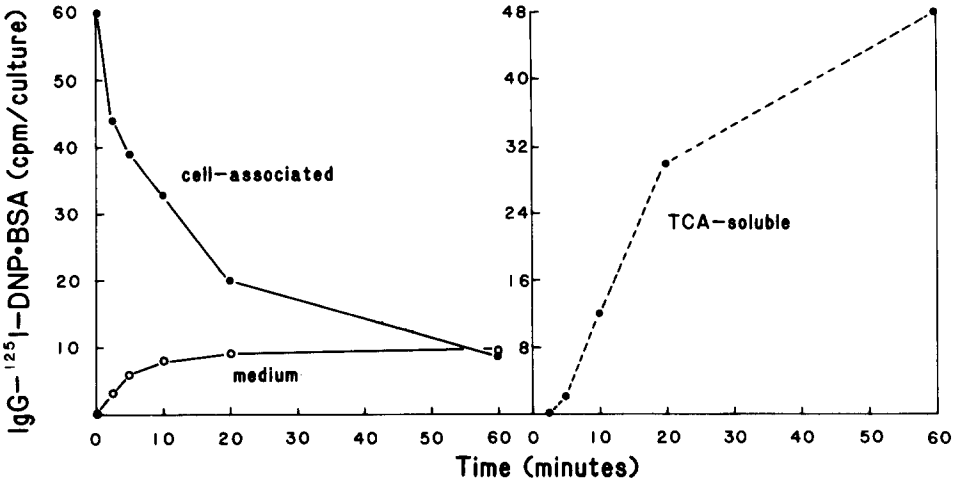

FIGURE 3 Internalization and degradation of IgG-[^{125}I]-DNP·BSA immune complexes. IgG complexes were formed using ^{125}I-labeled DNP·BSA (2.5:1 IgG to BSA ratio; 20 μg/ml IgG) and bound to J774 cells in 35-mm dishes (1×10^6 cells/dish) at 4°C. Cultures were warmed to 37°C and both culture medium and cells were harvested at the indicated time intervals. (*left*) Total cell-associated ^{125}I (●) was determined by dissolving monolayers in two 1-ml aliquots of 1% Triton X-100. TCA-precipitable ^{125}I released into the medium was also measured (10% TCA, 1 h, 4°C) (○). (*right*) The amount of the medium radiolabel that was acid soluble is shown. Degradation products of ^{125}I-DNP·BSA were detected over background (i.e., cells incubated with ^{125}I-IgG-complexes and 2.4G2) within 5 min of warming. TCA-soluble ^{125}I was judged to be mono-(or di-)iodotyrosine by $CHCl_3$ extraction (20).

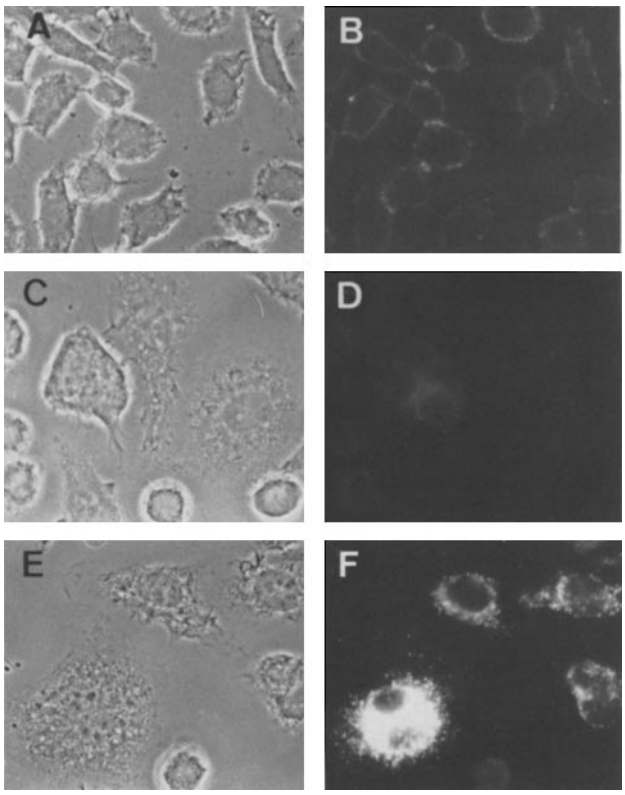

FIGURE 4 Visualization of IgG-DNP·BSA complexes by indirect immunofluorescence. J774 cells were incubated with 20 μg/ml IgG-complexes for 2 h at 4°C (*A* and *B*) or at 37°C (*C–F*) prior to fixation and staining with rhodamine-conjugated goat anti-rabbit IgG (see text). Cells in *E* and *F* were permeabilized with 0.1% Triton X-100 prior to staining. Fluorescence staining of cells incubated with ligand at 4°C was identical whether or not cells were Triton-treated. × 1,125.

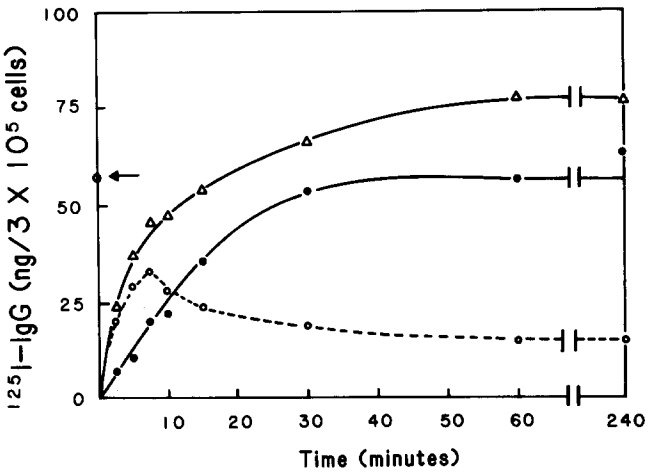

FIGURE 5 Uptake of ^{125}I-IgG-DNP·BSA at 37°C. Cells were exposed to 20 μg/ml ^{125}I-IgG complexes for the indicated time intervals, havested, and treated with subtilisin to distinguish surface-bound (○) from intracellular (●) radiolabel. The total amount of cell associated ^{125}I (△) was determined by adding the subtilisin-resistant and -released radioactivity. The amount of ^{125}I-IgG complexes bound to surface FcR at 4°C (2 h) is indicated by an arrow. All data corrected for nonspecific binding by inclusion of 2.4G2 in parallel cultures. Release of acid-soluble ^{125}I into the medium could not be detected over background during the time intervals shown.

sin-releasable). However, the amount of surface-bound ligand reached a peak within 7.5 min and decreased steadily thereafter. An apparent steady state occurred after 30 min, with the amount of ligand bound to surface FcR 20% of that bound at saturation at 4°C (Fig. 5, arrow). On the other hand, intracellular (subtilisin-resistant) ^{125}I-IgG accumulated somewhat more slowly until reaching a plateau after 30 min. Similar results were obtained for both J774 cells (Fig. 5) and primary cultures of peritoneal macrophages.

Degradation of Internalized Ligand

That the amount of intracellular ^{125}I-IgG remained constant for several hours (Fig. 5) suggested that a steady state had been reached between the rates of ligand internalization and digestion. Unfortunately, due to the slow rate of IgG degradation, the appearance of TCA-soluble radiolabel in the medium was difficult to detect over background in cells exposed to ^{125}I-IgG-DNP·BSA. As before, degradation was more easily studied when the BSA portion of the complex was labeled. As shown in Fig. 6, the incubation of J774 cells in IgG-[^{125}I]-

DNP·BSA was accompanied, after a 15-min lag, by release of TCA-soluble ^{125}I in the medium (presumably moniodotyrosine, as judged by $CHCl_3$ extraction [20]). Degradation continued for at least 4 h and was inhibited by 50% when 10 mM NH_4Cl was included in the incubation medium (not shown).

The relative rates of IgG and DNP·BSA degradation were estimated by relating the amounts of ^{125}I-IgG- or ^{125}I-DNP·BSA-complexes that were intracellular at the "steady state" (4 h, 37°C) to the respective amounts of ^{125}I-IgG- or ^{125}I-DNP·BSA bound to the cell surface at 4°C. Proportionately sixfold more ^{125}I-IgG than ^{125}I-DNP·BSA was present intracellularly (e.g., Fig. 5 vs. 6) suggesting that the DNP·BSA was degraded sixfold more rapidly. The rate of ^{125}I-IgG degradation was also measured directly. J774 cells were loaded with ^{125}I-IgG-complexes for 2 h at 37°C and then cultured in the absence of labeled ligand. Both the loss of cell-associated ^{125}I and the release of TCA-soluble ^{125}I into the medium proceeded with a half-time of 2–2.5 h in accord with previous measurements of IgG digestion by macrophages (5, 7). Thus, the $t_{1/2}$ of intracellular ^{125}I-DNP·BSA was estimated at 20–30 min (i.e., sixfold faster).

Internalization of Fc Receptors

While FcR mediate the efficient delivery of bound ligand to lysosomes, the receptors themselves are not efficiently reutilized. An examination of the data in Figs. 5 and 6 indicates that even after 4 h of incubation, J774 cells (and peritoneal macrophages) internalize and/or degrade an amount of ligand not much in excess of that accounted for by only one cell surface-equivalent of FcR: i.e., the amount of ligand bound to cells at saturation in the cold (1×10^6 molecules of IgG). To determine why macrophage FcR did not appear to mediate more than one round of ligand upake, we studied the internalization and intracellular fate of J774 FcR using specific monoclonal and polyclonal antireceptor antibodies.

As previously shown for the phagocytic uptake of IgG-coated particles (5), the pinocytosis of IgG complexes led to a selective removal of FcR from the macrophage plasma membrane. Following a 2-h exposure at 37°C to saturating concentrations of IgG complexes, the number of surface FcR was found to be decreased by two-thirds, as indicated by the decreased binding of ^{125}I-labeled Fab fragment of the antireceptor monoclonal antibody 2.4G2 (Table I). In contrast, the binding of several other ^{125}I-labeled monoclonal antibodies, directed against unrelated macrophage plasma membrane antigens, was unchanged. This includes the expression of another known macrophage receptor, that for the C3bi component of the complement pathway (recognized by monoclonal antibody 1.21J) (21). The decrease in ^{125}I-2.4G2 Fab binding was not due simply to competition by FcR-bound IgG-complexes. Identical results were obtained using cells treated with subtilisin (to remove any surface-bound complexes) prior to assay. Control experiments indicated that subtilisin treatment did not destroy the 2.4G2 Fab binding site.

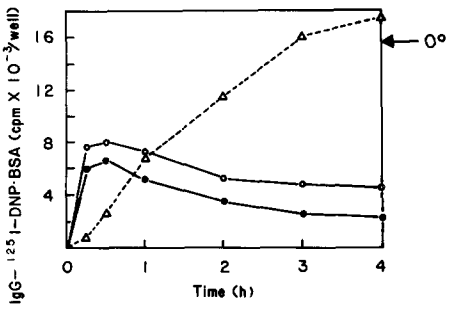

FIGURE 6 Uptake and degradation of IgG-^{125}I-DNP·BSA at 37°C; Experiment identical to that of Fig. 5 except that complexes were formed using ^{125}I-DNP·BSA. Subtilisin-releasable (○, surface-bound) and -resistant (●, intracellular) ^{125}I are shown, as well as the appearance of TCA-soluble radiolabel in the medium (△). After 4 h, the amount of intracellular radiolabel was <20% of that which could be bound to cells at saturation at 4°C (arrow). In contrast, the amount of intracellular radiolabel at 4 h using ^{125}I-IgG-labeled complexes (Fig. 5) was roughly equivalent to the amount of 4°C surface binding. See text for details.

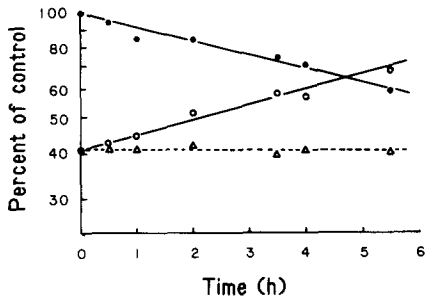

FIGURE 7 Recovery of surface FcR following reculture in IgG-complex-free medium. J774 cells were incubated in 20 μg/ml IgG complexes for 2 h at 37°C, washed, and then recultured in complex-free medium either in the absence (○) or presence (△) of 1 μg/ml cycloheximide. At the indicated times, surface FcR were quantitated by binding ^{125}I-2.4G2 Fab at 4°C. For comparison, binding to cycloheximide-treated cells not previously exposed to IgG-complexes is also shown (●). The number of ^{125}I-Fab binding sites decreased with a half-time of 8 h. If taken as a measure of FcR turnover, the rate obtained is some twofold more rapid than that measured in the absence of protein synthesis inhibitors by immunoprecipitation of labeled FcR (see Fig. 9).

This loss of surface FcR was largely irreversible. Upon reculture in IgG complexe free medium, the number of 2.4G2 Fab binding sites increased very slowly and after 5 h, was still <70% of control. Moreover, this increase was prevented by 1 μg/ml cycloheximide, suggesting that it resulted from the synthesis of new receptors (Fig. 7).

Fate of Internalized Receptors

Since IgG complexes were delivered to and degraded in lysosomes, it seemed likely that the removal of FcR from the plasma membrane was due to the degradation of internalized receptors. Accordingly, we measured the rate of FcR turnover in the presence and absence of ligand. For these experiments, J774 cells were radioiodinated at 4°C using lactoperoxidase-glucose oxidase, washed, plated, and then cultured at 37°C in medium with or without 20 μg/ml unlabeled IgG complexes. At various times thereafter, cells were havested (by scraping), lysed in Triton X-100, and ^{125}I-FcR quantified by immune precipitation using a specific rabbit anti-FcR antiserum that detects free receptor and receptor-ligand complexes with equal efficiency (5). An example of the immunoprecipitates obtained is shown in Fig. 8. In agreement with our previous

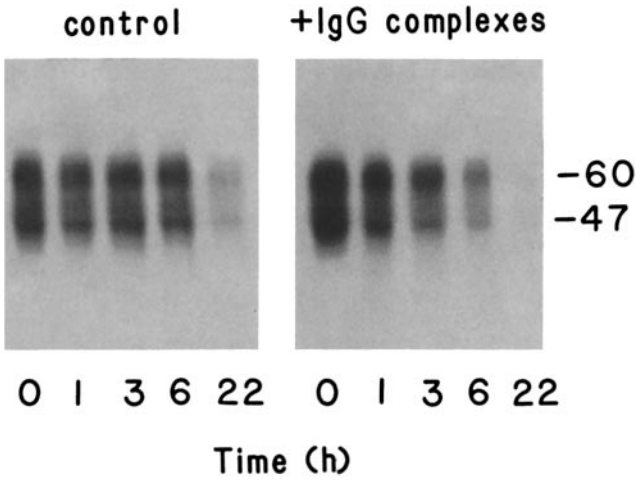

FIGURE 8 Immunoprecipitation of FcR from ^{125}I-labeled J774 cells incubated in the presence or absence of ligand. Cells were iodinated at 4°C and then incubated at 37°C in the presence or absence of 20 μg/ml IgG complexes. At the indicated time intervals, cells were harvested, lysed, and FcR immunoprecipitated using a specific rabbit anti-FcR antiserum. Immunoprecipitates were analyzed by electrophoresis on 4–11% SDS polyacrylamide gels that were then fixed, dried, and autoradiographed using Kodak XAR X-ray film and DuPont intensification screens (Lighting Plus).

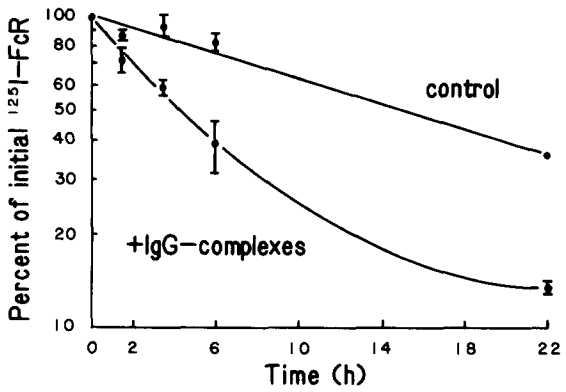

FIGURE 9 Turnover of ^{125}I-FcR in cells incubated in the presence or absence of ligand; Data from two paired experiments of the type shown in Fig. 8. Autoradiographs were used as templates to excise radiolabeled bands for quantitation.

results (17), FcR from J774 cells consisted of two somewhat diffuse bands of 60 and 47 kdaltons. These bands were cut out from dried gels and counted. As shown in Fig. 9 (data pooled from different experiments), the rate of ^{125}I-FcR degradation in control cells exhibited log-linear kinetics with a $t_{1/2}$ of 15 h. However, exposure of cells to saturating concentrations of IgG-complexes markedly accelerated receptor turnover such that the initial rate of degradation occurred with a $t_{1/2}$ ~5 h. This increased turnover was apparently selective for FcR, since the rate of degradation of another unrelated macrophage plasma membrane protein (the 90-kdalton glycoprotein recognized by the monoclonal antibody 2D2C) was similar ($t_{1/2}$ 20–22 h) in cells cultured in the presence and absence of IgG-complexes.

DISCUSSION

The results presented in this paper demonstrate that the binding of soluble IgG-immune complexes to macrophage FcR leads to the internalization and degradation of both ligand and receptor. Thus, they confirm and extend our previous studies of FcR-mediated phagocytosis of IgG-coated particles (5). In both cases, specific antireceptor antibodies were used to document the selective removal of FcR from the plasma membrane and its degradation following delivery to lysosomes. Accordingly, upon the binding of ligand, the intracellular fate of the macrophage FcR is the same irrespective of its mode of entry: phagocytosis or, in the case of IgG complexes, coated vesicle-mediated pinocytosis (2).

The present findings have their greatest significance when viewed in the context of the accompanying paper (3) in which we studied the internalization and fate of FcR tagged with a monovalent Fab fragment of the antireceptor antibody 2.4G2. It was shown that Fab-FcR complexes were internalized and avoided degradation by rapidly recycling to the cell surface. Moreover, Fab-FcR complexes did not reach lysosomes, suggesting that the recycling of internalized receptors occurred from prelysosomal endosomes (4). The pinocytosis of IgG-complexes was, in contrast, not associated with the recycling of internalized FcR to the plasma membrane. The fact that both receptor and ligand were degraded suggests that the receptor was routed to lysosomes. Indeed, internalized FcR-bound ^{125}I-IgG-complexes have recently been found to cosediment with a high density hydrolase-rich fraction after centrifugation in Percoll density gradients (P. Ukkonen, A. Helenius, and I. Mellman, unpublished results). These findings suggest that interaction of macrophage FcR with a polyvalent ligand removes the receptor from a constitutive recycling pathway and causes its transport to lysosomes.

Little is known about the molecular signals that control the traffic of receptors and other membrane components during endocytosis. A number of plasma membrane receptors, such as the FcR and receptors for epidermal growth factor and insulin (22–25), seem to interact with their ligands in a way that signals the transport of receptors from the cell surface to some intracellular compartment(s). In the case of FcR, this alteration in traffic appears to be accomplished by the cross-linking or aggregation of adjacent receptors attached to multivalent ligand. Thus, FcR bound by IgG-complexes do not recycle after internalization whereas FcR tagged with monovalent antireceptor Fab fragments do (3). Alternatively, the differences in the behavior of the FcR when bound by antireceptor Fab on the one hand, and IgG complexes on the other, may have less to do with the valency than with the nature of the ligand employed. While the Fab binds to FcR via its antigen combining site, the IgG-complex is a physiological ligand that binds via the Fc domains of intact IgG molecules. It is possible that the binding of the natural ligand may itself cause a conformational (or other) change in the receptor that could account for any subsequent changes in its intracellular fate. Unfortunately, it has thus far been difficult to study the behavior of FcR bound to the Fc portion of monomeric IgG, since this ligand binds with low affinity (6, 14). However, the experiments using a second F(ab')$_2$ antibody to cross-link receptor-bound Fab suggest that simple aggregation of FcR may be sufficient (3).

Pathway of FcR-mediated Pinocytosis

Recent study using IgG complexes coupled to colloidal gold has indicated that the pinocytosis of FcR-bound ligands proceeds by their accumulation at clathrin-coated regions of the

plasma membrane followed by internalization in coated vesicles. Shortly thereafter, the IgG-complexes appear in a heterogeneous population of uncoated endocytic vacuoles, endosomes, prior to their delivery to electron-dense lysosomes and autophagic vacuoles (2, 9). Thus, it appears that IgG complexes are internalized along the same general pathway as many other ligands of receptor-mediated pinocytosis (26).

The importance of endosomes has recently been appreciated due, in part, to a number of observations that indicate that these vacuoles have an acidic internal pH (4, 27–30). Many ligands, including mannose- and mannose-6-phosphate-containing glycoproteins (31, 32) and asialoglycoproteins (33), bind to their receptors in a pH-sensitive fashion. Upon reaching endosomes, these ligand-receptor complexes should dissociate and generate free receptors that are then available to reteurn to the cell surface (4). In contrast, the binding of IgG complexes to FcR is not sensitive to low pH. Thus, the ligand-receptor complex may remain intact, cross-linking receptors on the endosome membrane.

Receptor Clustering and Receptor Recycling

It is not clear why the cross-linking of FcR by multivalent ligands should result in the transport of receptors to lysosomes. Receptor clustering may provide a signal for the selective transport of receptor-ligand complexes from endosomes to lysosomes. We favor an alternative possibility, however, that receptor aggregation prevents the inclusion of receptor-ligand complexes into nascent recycling vesicles that continuously leave the endosome bound for the plasma membrane. Cross-linked FcR may be transferred to a hydrolytic compartment as the endosome moves into the perinuclear region and fuses with lysosomes (4, 34–36).

Conceivably, clustering may be of importance in regulating the traffic of a variety of receptors and nonreceptor membrane proteins. For example, Anderson et al. (37) have found that incubation of fibroblasts with polyclonal antibody against the low density lipoprotein receptor leads to the loss of receptor from the plasma membrane, presumably by preventing recycling. Monovalent antibody does not have this effect. Similarly, epidermal growth factor receptor is removed from the surfaces of cells incubated with a pentameric monoclonal antireceptor IgM, but not its monovalent Fab fragment (38).

Most receptors are believed to accumulate at clathrin-coated pits on the plasma membrane after binding specific ligand (1, 26). Whether recruitment to coated pits is due to cross-linking by multivalent ligands, intermolecular interactions among the receptors themselves, or interactions between receptors and coated pit components is not known. The paradigm suggested here would require that these receptors must disaggregate soon after internalization, presumably in endosomes, for recycling to occur. Disaggregation may be at least partly facilitated by the acidic internal pH of the endosome that in many cases results in the dissociation of receptor-ligand complexes. Receptors bound to aggregating ligands, which do not come off at low pH may thus be prevented from recycling, resulting in their transport to and degradation in lysosomes.

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