

Internalization and Rapid Recycling of Macrophage Fc Receptors Tagged with Monovalent Antireceptor Antibody: Possible Role of a Prelysosomal Compartment

IRA MELLMAN, HELEN PLUTNER, and PENTTI UKKONEN

Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510

ABSTRACT Binding and pinocytosis of polyvalent IgG-containing immune complexes by mouse macrophages leads to the selective removal of Fc receptors (FcR) from the cell surface and to the rapid delivery of receptor and ligand to lysosomes, where both are degraded (I. Mellman and H. Plutner, 1984, *Journal of Cell Biology*, 98:1170–1177). In this paper, we have studied the internalization of FcR tagged with a monovalent probe that, unlike IgG-complexes, cannot cross-link adjacent receptors. We have used an Fab fragment of high affinity anti-FcR monoclonal antibody whose binding was completely sensitive to low pH (4.0) at 4°C. Thus, surface-bound (acid-releasable) and intracellular (acid-resistant) ¹²⁵I-Fab could be readily distinguished. Incubation of J774 macrophages with ¹²⁵I-Fab at 37°C did not lead to the accumulation of large amounts of the antibody in the acid-resistant compartment. After 3 h, only 20% of the total cell-associated radiolabel was intracellular. The internalized ¹²⁵I-Fab was also shown by Percoll gradient centrifugation to be associated primarily with low density endosomes, as opposed to lysosomes. Significantly, most of the labeled antibody returned rapidly to the plasma membrane, still bound to FcR. This recycling was complete within 10 min, was unaffected by NH₄Cl, and was only slightly inhibited by the Na⁺-H⁺ ionophore monensin.

These results indicate that monovalent Fab-FcR complexes are internalized, delivered to endosomes, and rapidly returned to the cell surface. Since the internalization of polyvalent IgG-complexes removed the FcR from this recycling pathway and caused its transport to lysosomes, we suggest that the state of receptor aggregation in the endosome membrane helps determine its intracellular fate.

Although endocytosis in animal cells is generally accompanied by the return (or recycling) of internalized plasma membrane to the cell surface, several plasma membrane receptors are known whose activities are largely if not irreversibly removed from the cell surface during ligand uptake (1). Classically, this phenomenon is illustrated by the “down regulation” of receptors for certain polypeptide hormones (e.g., insulin, epidermal growth factor) in cells exposed to hormone. In most cases, the mechanism of receptor loss is unknown. One well-studied example, however, is the mouse macrophage receptor for the Fc domain of immunoglobulin G (IgG). The Fc receptor

(FcR)¹ is a membrane glycoprotein, consisting of two polypeptides (60 and 47 kdaltons) (2, 3), which can mediate the phagocytosis of large IgG-coated particles as well as the pinocytosis of soluble antibody-antigen complexes (1, 4, 5). In recent work (see accompanying paper [4] and reference 5), we have used specific anti-FcR antibodies to show that the uptake of either of these multivalent ligands leads to the rapid

¹ *Abbreviations used in this paper:* FcR, Fc receptors; α MED, α -modified Eagle's medium; PBS-G, PBS containing 5 mM glucose; TCA, trichloroacetic acid; α 8, α MED containing 8% serum.

internalization and intralysosomal degradation of the receptor.

In this paper, we show that FcR tagged with a monovalent ligand behaves quite differently. A high affinity Fab fragment of a monoclonal antireceptor antibody was used which could not cross-link adjacent FcR in the plane of the membrane. In contrast to the results obtained using multivalent IgG-complexes, the Fab-FcR complex was internalized and recycled intact to the cell surface.

MATERIALS AND METHODS

Cell and Cell Culture: The macrophage cell line J774 (6) was grown in suspension culture in α -modified Eagle's medium (α MEM) supplemented with 4% fetal calf serum (FCS; heat inactivated at 56°C for 30 min) and penicillin-streptomycin. For experiments, 4×10^5 cells were plated in 16-mm wells (24-well tissue culture dishes; Costar, Cambridge, MA) for 1 h at 37°C in α MEM containing 8% serum (α 8). Monolayers were rinsed two times with PBS to remove any nonadherent cells prior to use.

Antibodies: The monoclonal rat anti-mouse macrophage FcR antibody 2.4G2 IgG was produced, grown as ascites in mice, and purified as described (7). Fab fragments, generated using papain (Sigma Chemical Co., St. Louis, MO), were purified by DEAE-cellulose chromatography (8). All antibody preparations used were essentially pure, as judged by SDS PAGE. 2.4G2 Fab was labeled using Na¹²⁵I (Amersham Corp., Arlington Heights, IL) and Iodogen (Pierce Chemical Co., Chicago, IL) as described (9). Antibody was separated from unincorporated ¹²⁵I by chromatography on Dowex 1-X8 (200–400 mesh; Sigma Chemical Corp.) (5) and stored for up to 5 wk at 4°C in PBS containing 1 mg/ml BSA and 0.02% NaN₃. The antibody's specific radioactivity varied from 0.5 – 3.3×10^6 cpm/ μ g protein. Rabbit anti-rat IgG (Pel-Freeze Biologicals, Rogers, AR) was purified by affinity chromatography and F(ab')₂ fragments were generated using pepsin (Sigma Chemical Co.) as described (5).

pH Elution Assay: Cells were exposed to saturating concentrations of ¹²⁵I-2.4G2 Fab (1 μ g/ml) at 4° or 37°C in medium containing 10 mM HEPES (pH 7.2; α 8-H). Nonspecific binding, uptake, and degradation were determined in parallel wells by including 100 μ g/ml unlabeled 2.4G2 which routinely inhibited ¹²⁵I-binding by >95%. Following incubation, cells were placed on ice and washed four times with cold PBS containing 5 mM glucose (PBS-G). Acid-releasable (presumably cell surface-associated) ¹²⁵I-Fab was determined at 4°C by treating the monolayers twice with 0.5 ml of serum-free α -H, adjusted with HCl to pH 4.0, for 5 min each time. Both acid washes were pooled and ¹²⁵I was determined in a gamma scintillation spectrometer. After one additional rinse with 0.5 ml pH 4.0 medium, cell-associated radioactivity was determined by harvesting monolayers with a cotton-tipped swab. Alternatively, the cells were returned to culture in complete medium (neutral pH) and subjected to a second round of pH 4.0 elution at various times thereafter. Neither cell viability nor morphology was affected by this procedure. Elution of surface-bound ¹²⁵I-Fab was extremely efficient, with >97% of the radiolabel removed from cells that had been incubated with the antibody for 1 h at 4°C (at which temperature, little or no endocytosis should have occurred).

Subcellular Fractionation: After washing, J774 cells were homogenized in cold 0.25 M sucrose containing 2 mM EDTA and 10 mM HEPES (pH 7.4) using a stainless steel homogenizer (Kontes Co., Vineland, NJ). A postnuclear supernatant solution (750 g, 10 min, 4°C) was diluted with Percoll (Sigma Chemical Co.) to a final Percoll concentration of 27% (vol/vol) and centrifuged in a Beckman Ti70 rotor (20,000 g, 2 h, 4°C; Beckman Instruments, Inc., Palo Alto, CA) as described (10). Recoveries of ¹²⁵I-Fab were typically >90%. β -glucuronidase activity was measured in the presence of 0.1% Triton X-100 as described (11).

RESULTS

Binding and Endocytosis of 2.4G2 Fab

The monoclonal antibody 2.4G2 was originally selected because it can inhibit macrophage rosetting of IgG-coated erythrocytes (7). Although macrophages and lymphocytes may express more than one class of FcR, the affinity ($K_A > 10^9$ M⁻¹) and specificity of this antibody for a trypsin-resistant FcR that selectively binds IgG2b/IgG1-containing immune complexes has been well documented (2, 3, 7). Moreover, 2.4G2 Fab has been used as an affinity adsorbent to purify

biologically active receptor from detergent lysates of J774 cells (7). Thus, it can be presumed that the only antigen recognized by 2.4G2 in mouse macrophages is the FcR.

At 4°C, ¹²⁵I-2.4G2 Fab bound rapidly to J774 cells; saturation was reached within 15–30 min. As shown in Fig. 1, virtually all (>98%) of this bound antibody was removed by brief treatment with pH 4 medium in the cold. Given a 1:1 stoichiometry between the binding of a monoclonal Fab fragment to its antigen, we estimate that under these conditions the amount of antibody bound corresponded to 7 – 8×10^5 FcR/cell. ¹²⁵I-Fab binding was inhibited >95% by simultaneous incubation with 100-fold excess of unlabeled 2.4G2 IgG.

After 3 h at 37°C, the amount of cell-associated ¹²⁵I-Fab was 1.5–2 times greater than at 4°C. Whereas most of the bound antibody was still acid-releasable, a significant amount could not be eluted and was presumably intracellular (Fig. 1). Within 15 min, 8% of the total radioactivity was acid-resistant; this amount increased to 20–25% over the next 3 h. In contrast, an increase in acid-resistant binding was not observed when ¹²⁵I-Fab was incubated with a crude preparation of J774 membranes at 4° and 37°C (not shown). Thus, the Fab does not simply bind to FcR in an acid-resistant fashion at 37°C: intact cells (and presumably internalization of the antibody) are required.

After a continuous incubation with ¹²⁵I-Fab for 3 h, little or no trichloroacetic acid (TCA)-soluble ¹²⁵I (presumably as monoiodotyrosine) was detected in the growth medium. However, even after CHCl₃ extraction of the acid soluble fraction (12), there was a significant background of TCA-soluble ¹²⁵I that would have made it difficult to detect a slow rate of digestion.

Recycling of 2.4G2 Fab-FcR Complexes

The results of Fig. 1 suggest that the Fab-FcR complex may be continuously internalized and returned intact to the cell surface. To test this possibility, we determined whether any of the intracellular (i.e., acid-resistant) ¹²⁵I-Fab could reappear

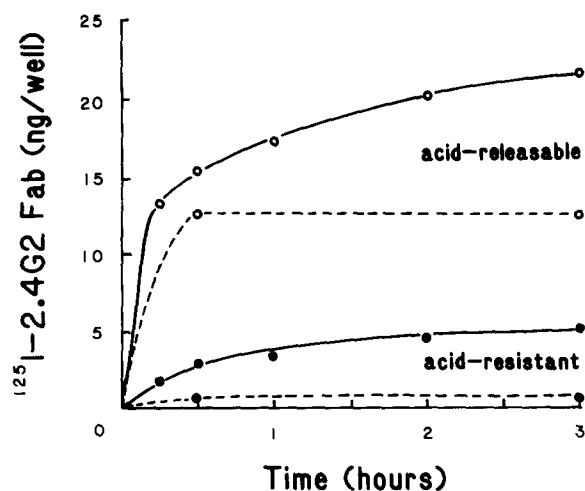


FIGURE 1 Low pH-mediated release of ¹²⁵I-2.4G2 Fab from J774 cells. J774 monolayers were incubated with ¹²⁵I-2.4G2 Fab at 4° (---) or 37°C (—) for the indicated time intervals. Following incubation, the cells were washed with PBS-G at 4°C to remove unbound radiolabel and treated for 10 min with pH 4.0 α MEM-H in the cold. Acid-releasable (O, surface bound) and acid-resistant (●, intracellular) ¹²⁵I-Fab was determined as described in the text.

on the cell surface (i.e., become acid-sensitive). Cells were incubated for 1 h at 37°C with ^{125}I -2.4G2 Fab, washed with cold PBS-G, and treated at 4°C with pH 4.0 medium to remove surface bound antibody (see Materials and Methods). Cultures were then fed with warm $\alpha 8\text{-H}$ and maintained at 37°C for various lengths of time. A second low pH treatment in the cold was used to determine surface-bound and intracellular ^{125}I . Control cultures were kept at 4°C following the initial low pH wash.

As shown in Fig. 2, a significant fraction (45–73% in various experiments) of the internalized ^{125}I -Fab became accessible to low pH release upon continued incubation. Particularly striking was the rapidity of the process: the reappearance of ^{125}I -Fab on the cell surface was complete within 10 min ($t_{1/2}$ of 3 min) at 37°C. Relatively little ^{125}I was released into the medium (5% of the total during the first 10-min period, 20% in 40 min), most of which was TCA-soluble, indicating that a small fraction of internalized antibody was in fact degraded. In contrast, if cells were incubated at 4°C, only a small fraction of the total cell associated ^{125}I became acid-sensitive (Figs. 2 and 3).

The extent to which ^{125}I -Fab reappeared on the plasma membrane depended partly on the length of time the cells had initially been incubated with the antireceptor antibody. As the duration of the initial exposure to ^{125}I -Fab increased from 15 min to 2 h, the fraction of internalized radiolabel that became acid-releasable after 15 min of reculture at 37°C decreased from 73 to < 60% (Fig. 3). Thus, with time, ^{125}I -Fab accumulated in an endocytic compartment from which rapid recycling does not occur.

Effects of NH_4Cl and Monensin

The reappearance of ^{125}I -Fab on the cell surface was largely

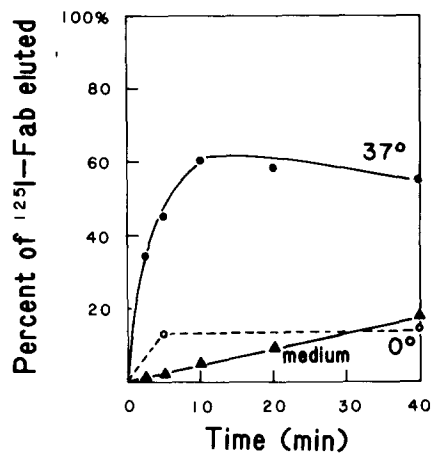


FIGURE 2 Reappearance of internalized ^{125}I -2.4G2 Fab on the plasma J774 cells were incubated for 1 h at 37°C in medium containing ^{125}I -Fab and then rapidly cooled on ice and washed with PBS-G in a coldroom. Following treatment with cold pH 4 $\alpha\text{MEM-H}$ to remove surface-bound ^{125}I -Fab, the cells were fed with warm $\alpha 8\text{-H}$ and cultured at 37° or 0°C. At the indicated time intervals, the medium (\blacktriangle) was harvested, and the cells were washed and subjected to a second-pH 4 treatment in the cold to assess the reappearance of ^{125}I -Fab on the cell surface. Acid-releasable radiolabel is given as a percentage of total ^{125}I -Fab (acid-releasable, cell-associated, and medium) at each time point. At 37°C, most of the ^{125}I -Fab that had been acid-resistant at time became acid-releasable following 10 min of culture (\bullet). Little ^{125}I -Fab became acid-sensitive in cells cultured at 0°C (\circ).

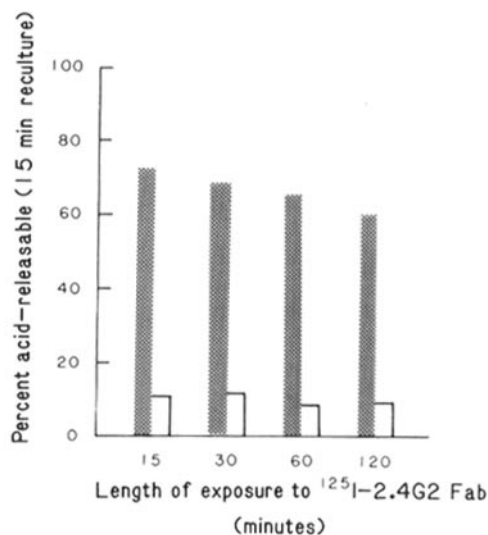


FIGURE 3 Reappearance of Fab-FcR complexes on the cell surface following different lengths of exposure to ^{125}I -2.4G2 Fab. J774 cells were incubated with ^{125}I -Fab at 37°C (stippled bars) for 15–20 min, acid-stripped, and cultured at 37° or 0°C (open bars) in Fab-free $\alpha 8\text{-H}$. 15 min later, the percent of ^{125}I -Fab that was acid-releasable was determined.

insensitive to the effects of two agents that are thought to interfere with plasma membrane or receptor recycling in some systems (13–15). Inclusion of 10 mM NH_4Cl both during the initial 1-h exposure to ^{125}I -2.4G2 Fab and the subsequent reculture period affected neither the speed nor the extent to which intracellular ^{125}I became acid-sensitive. The binding of ^{125}I -Fab to surface FcR was also unaffected (not shown). However, the degradation of internalized Fab to TCA-soluble radioactivity was totally inhibited (Fig. 4). Similarly, treatment with the $\text{Na}^+\text{-H}^+$ ionophore monensin (25 μM) blocked degradation without dramatically affecting the reappearance of internalized ^{125}I -Fab. A slight (15–20%) but reproducible inhibition was observed (Fig. 4). 2-h incubation of J774 cells in 25 μM monensin (in the absence of added Fab) also decreased the number of surface FcR by 20%. Cycloheximide (1 $\mu\text{g}/\text{ml}$) was without any effect on either the internalization or surface reappearance of ^{125}I -Fab.

pH-Dependence of 2.4G2 Fab-FcR Dissociation

The evidence discussed thus far indicates that little ^{125}I -2.4G2 Fab accumulates intracellularly at 37°C. Instead, internalized Fab remains bound to FcR and apparently returns to the cell surface. In contrast, the internalization of other low pH-sensitive ligand-receptor complexes usually results in the discharge of ligand in some acidic intracellular compartment (e.g., lysosomes of prelysosomal endosomes) and accordingly in the continuous accumulation of the ligand in lysosomes (1, 16, 17). Since many of these ligands can dissociate from their receptors at a relatively high pH (5.5–6.0), we investigated the pH dependency of the 2.4G2 Fab-FcR interaction to determine why the Fab was not discharged from the FcR upon reaching endosomes or lysosomes.

As shown in Fig. 5, the dissociation of surface-bound ^{125}I -Fab at 4°C was virtually complete at pH < 4.3 and relatively unaffected (15% dissociated) at pH > 5.5. Half-maximal dissociation occurred at pH 4.7–4.8, an acidity similar to that determined for macrophage and J774 cell lysosomes using

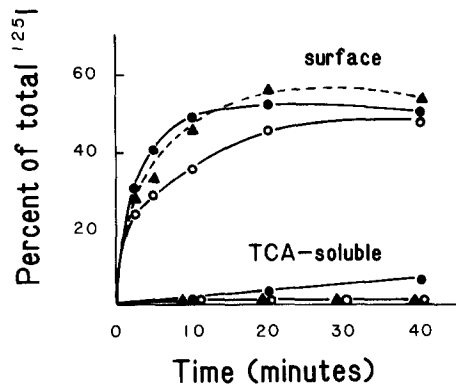


FIGURE 4 Effect of NH_4Cl and monensin on the reappearance of ^{125}I -2.4G2 Fab. Cells were incubated for 1 h at 37°C in $\alpha 8\text{-H}$ containing ^{125}I -Fab with or without 10 mM NH_4Cl or 25 μM monensin. Following acid-stripping in the cold, cells were cultured in Fab-free medium (containing the appropriate inhibitor) for 10–60 min before being subjected to a second round of low pH treatment. Acid-releasable ^{125}I -Fab is shown as a percent of total in control cells (●) and cells in NH_4Cl (▲) or monensin (○). The release of TCA-soluble radiolabel into the medium during the reculture period is also given as a percent of total ^{125}I at each timepoint.

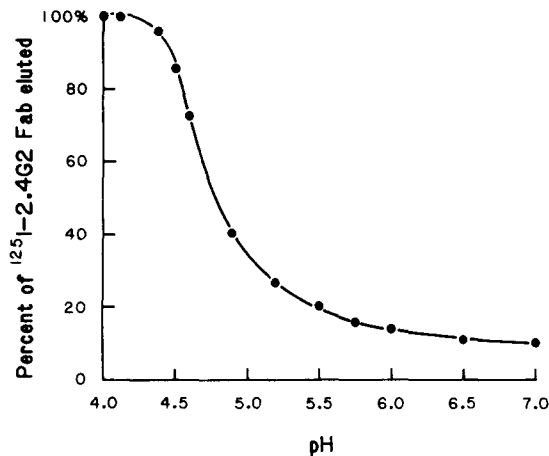


FIGURE 5 pH-dissociation profile of ^{125}I -2.4G2 Fab from J774 FcR. Cells were incubated with ^{125}I -Fab for 1 h at 4°C , washed with cold PBS-G to remove unbound antibody, and then treated with cold $\alpha\text{MEM-H}$ adjusted to the indicated pH's for 10 min (see Materials and Methods). The amount of ^{125}I -Fab released at each pH is expressed as a percentage of the amount released by treatment at pH 4.0 (at which pH, >98% of the total cell-associated radiolabel was removed).

fluorescein dextran fluorescence (10, 18). The binding of ^{125}I -Fab to cells was similarly inhibited by acidic pH. However, the inhibition was reversible: cells or ^{125}I -Fab treated for 15 min (4°C) at pH 4 would exhibit normal binding when the pH was returned to neutral.

The rates of dissociation at 37°C were also determined at pH 4.0 and at various pH's that may occur intracellularly. Fig. 6 summarizes experiments in which cells were exposed to ^{125}I -Fab at 37°C for 1 h, washed, and then incubated at 37°C in $\alpha\text{MEM-H}$ adjusted to the indicated pH's. Similar results were obtained at 4°C . The amount of ^{125}I -Fab eluted at any given pH was expressed as a fraction of the maximum, i.e., that which was eluted by treatment with pH 4.0 medium for 10 min. Although elution at pH 4.0 was clearly the most efficient ($t_{1/2} < 0.5$ min), dissociation at pH 4.6 was neverthe-

less rapid. 50% of the cell-associated Fab was removed in <1.5 min. When expressed in this way, half-maximal dissociation at pH 4.8 required ~ 3 min. 50% dissociation was never reached at pH >5.0 in these experiments. Taken together, these results indicate that significant and rapid disruption of the Fab-FcR complex occurs at pH's that approximate the pH found in secondary lysosomes, but dissociation is greatly reduced at pH's only slightly higher. Thus, the Fab-FcR complex may not enter, or may reside only briefly, in a compartment of sufficiently low pH, such as lysosomes, to mediate discharge of the Fab from FcR.

Subcellular Localization of Internalized 2.4G2 Fab

To examine the subcellular localization of internalized ^{125}I -Fab, cell homogenates were centrifuged in Percoll density gradients (10). J774 cells were incubated with ^{125}I -Fab for 0.5–1.5 h at 37°C and treated with low pH medium in the cold to remove surface-bound radiolabel prior to homogenization. As shown in Fig. 7B, most of this acid-resistant ^{125}I -Fab sedimented as a low density peak ($\rho = 1.03\text{--}1.04$) that was well resolved from a much higher density peak ($\rho = 1.10$) that contained the lysosomal marker enzyme β -glucuronidase (Fig. 7A). Only a small amount of ^{125}I -Fab (7–12% in various experiments) co-sedimented with the high density lysosome fraction. Thus, the bulk of the Fab internalized under these conditions was associated with a nonlysosomal endosome fraction.

The presumptive Fab-containing endosomes sedimented at a density that was slightly heavier than that of J774 plasma membranes (marked by ^{125}I -Fab bound to cells at 4°C) (Fig. 7A). Although not well resolved under gradient conditions used in Fig. 7, these two fractions were more clearly separated

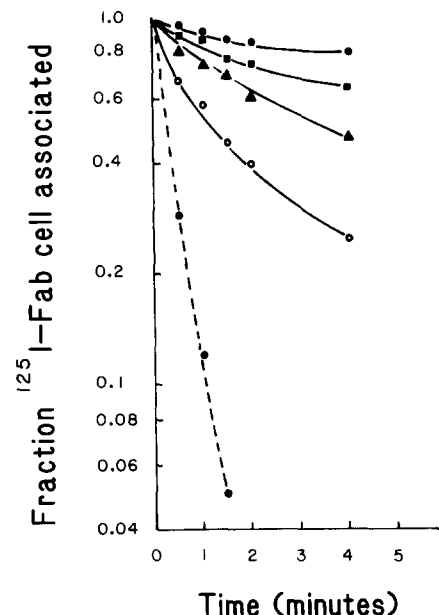


FIGURE 6 Time course of ^{125}I -2.4G2 Fab dissociation from J774 FcR at different pH's. Cells were exposed to ^{125}I -Fab for 30 min at 37°C , washed, and then incubated in $\alpha\text{MEM-H}$ adjusted to the indicated pH's for 0.5–10 min, also at 37°C . The amount of ^{125}I -Fab released at each timepoint is expressed as one minus the fraction of the maximum amount released (i.e., after 10 min at pH 4.0). Identical results were obtained using cells incubated at 4°C . ●, pH 5.5; ■, pH 5.0; ▲, pH 4.8; ○, pH 4.6; ●-●, pH 4.0.

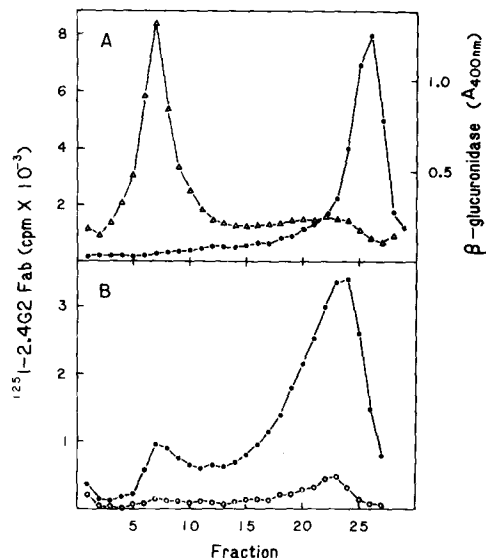


FIGURE 7 Sedimentation of cell surface-bound (A) and internalized ^{125}I -2.4G2 Fab (B) in Percoll density gradients. (A) A confluent 100-mm dish of J774 cells ($\sim 1 \times 10^7$ cells) was incubated in ^{125}I -Fab for 60 min at 4°C , washed, and harvested using a Teflon scraper. These cells were combined with $\sim 4 \times 10^7$ unlabeled carrier cells prior to homogenization and centrifugation in 27% Percoll (10). Cell surface-bound ^{125}I -Fab sedimented as a symmetrical low density peak ($\rho = 103$ g/ml) (●) well separated from the peak of the lysosome marker enzyme β -glucuronidase ($\rho = 1.10$ g/ml) (Δ). (B) Cells were incubated with ^{125}I -Fab for 60 min at 4° or 37°C and treated with pH 4.0 medium to remove surface-bound radiolabel prior to harvest. Most (90%) of the acid-resistant (internalized) ^{125}I -Fab in the 37°C cells (●) sedimented as a low density, asymmetrical peak that was slightly heavier than that of cell surface-bound ^{125}I -Fab (A). A small amount of radiolabel co-sedimented with high density lysosomal markers. For comparison, the sedimentation profile of acid-resistant radiolabel in cells incubated with ^{125}I -Fab at 4°C is shown (○). The amount of cell associated ^{125}I present before acid stripping was equivalent in both the 37° and 4°C samples.

by subjecting the low density region of the gradient to a second centrifugation (19) or by centrifuging the initial homogenate in lower concentrations of Percoll (7.5–10%) (P. Ukkonen and I. Mellman, unpublished results). In addition, when cells that had bound ^{125}I -Fab at 4°C were treated with low pH medium prior to homogenization, little radiolabel sedimented in the low density fractions (Fig. 7B).

To determine whether the clustering of monovalent Fab-FcR complexes alters their intracellular fate, we studied the subcellular localization of ^{125}I -Fab internalized in the presence of a cross-linking second antibody, affinity purified $\text{F}(\text{ab}')_2$ fragments of a rabbit anti-rat IgG. $\text{F}(\text{ab}')_2$ fragments were necessary to prevent the second antibody from binding to FcR via its Fc domain. Although the degree of cross-linking obtained was not extensive (binding experiments showed that on average, only one ^{125}I - $\text{F}(\text{ab}')_2$ was bound per Fab), the second antibody caused a significant increase in the transport of ^{125}I -Fab to high density lysosomes. In the experiment shown in Fig. 8, J774 cells were incubated in α 8-H containing ^{125}I -Fab ($1 \mu\text{g}/\text{ml}$) for 1 h at 17°C , washed briefly, and then incubated for an additional 1 h at 17°C with or without saturating concentrations ($50 \mu\text{g}/\text{ml}$) of the anti-rat $\text{F}(\text{ab}')_2$. In this way, the endosome compartment was loaded with both antibodies, as at temperatures $<20^\circ\text{C}$, FcR-bound ligands are arrested in endosomes (P. Ukkonen, A. Helenius, and I.

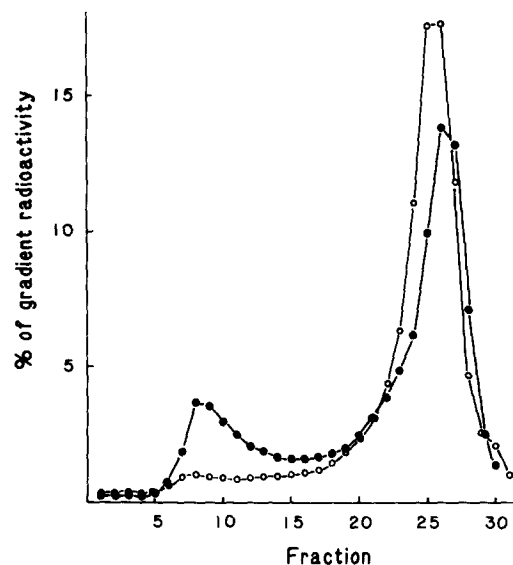


FIGURE 8 Effect of second antibody, rabbit anti-rat $\text{F}(\text{ab}')_2$, on the sedimentation pattern of cell-associated ^{125}I -2.4G2 Fab in Percoll density gradients. J774 cells were incubated with ^{125}I -Fab ($1 \mu\text{g}/\text{ml}$) for 1 h at 17°C , washed with medium, and incubated in α 8-H with (●) or without (○) saturating concentrations of rabbit anti-rat- $\text{F}(\text{ab}')_2$ ($50 \mu\text{g}/\text{ml}$) for 1 h at 17°C . The plates were then placed in a 37°C incubator for an additional hour. The cells were washed with cold PBS, homogenized, and centrifuged in 27% Percoll as in Fig. 7. Lysosomes were localized by β -glucuronidase activity, which was maximum at fractions 5 to 11 (peak at fraction 8). Unlike in Fig. 7, no acid wash was performed prior to homogenization; thus, both surface bound and intracellular ^{125}I -Fab are shown.

Mellman, unpublished results). The cultures were then shifted to 37°C for 1 h to permit transport to lysosomes. Following several washes in cold PBS, the cells were homogenized and centrifuged in Percoll. Unlike in Fig. 7B, however, no acid wash was performed prior to homogenization, since the presence of the second antibody greatly reduced the efficiency of removing surface-bound Fab. As shown in Fig. 8, the second antibody induced a small but significant increase in the amount of ^{125}I -Fab reaching lysosomes: 16% of the total (surface plus intracellular) radioactivity was found in the heavy density fractions as opposed to only 3% in the control gradient. Similar results were obtained when cells were incubated with both antibodies at 37°C throughout.

DISCUSSION

We have provided direct evidence for the internalization and rapid recycling of macrophage FcR through the use of a Fab fragment of the antireceptor monoclonal antibody 2.4G2. Given the acid lability of the Fab-FcR interaction at 4°C , we could distinguish surface-bound (acid-releasable) from intracellular (acid-insensitive) Fab and also follow the "reappearance" of interiorized antibody on the plasma membrane by incubating acid-stripped cells at 37°C . Little of the internalized Fab was degraded or released into the medium, indicating that most of the antibody remained bound to FcR during its transit through the vacuolar system. As a monovalent probe, the Fab was not likely to have "triggered" FcR internalization by cross-linking or otherwise aggregating receptors in the plane of the membrane. Nor did the Fab alter the rate of fluid pinocytosis, which was measured using horseradish peroxidase (I. Mellman, unpublished data). Accordingly, the inter-

nalization and subsequent reappearance of the Fab suggests a continuous internalization and recycling of FcR, consistent with the concept that pinocytosis is accompanied by the internalization and recycling of many plasma membrane proteins (1, 16, 20–22). It is also in agreement with our previous finding that pinocytic vesicles (or endosomes), iodinated intracellularly following the uptake of lactoperoxidase, contained labeled FcR (23). Thus, the long half life of J774 FcR (15 h) (4) apparently results not from the receptor's exclusion from nascent pinocytic vesicles but from its ability to recycle following internalization.

Kinetics of FcR Recycling

Both the amount of antibody internalized and the rate at which it returned to the cell surface agree with what is known about the quantitative aspects of membrane flow during pinocytosis. Peritoneal macrophages and J774 cells continuously internalize large areas of plasma membrane: approximately two cell surface equivalents of membrane are interiorized per hour (23, 24). When visualized using markers of fluid phase pinocytosis, pinocytic vesicle contents were transported sequentially to two identifiable endocytic compartments, endosomes and secondary lysosomes. Stereological measurements indicate that these compartments are roughly equivalent in size and together constitute 25–30% of the macrophage's surface area (24). Thus, assuming that every 30 min the entire macrophage cell surface passes through an intracellular compartment(s) no more than 25% its size, internalized membrane must return to the cell surface with great rapidity, approximately every 7.5 min. This estimate agrees well with the rate observed for the reappearance of intracellular Fab (Fig. 2). However, it is not yet known whether a membrane-bound marker such as the Fab is localized entirely to the same endocytic vesicles as markers of fluid pinocytosis. Nevertheless, even after prolonged incubation at 37°C, no more than 20% of the total cell associated ¹²⁵I-Fab was resistant to removal by low pH, suggesting (perhaps coincidentally) that the Fab containing compartment may also have a membrane area ~20% of the cell surface.

Pathways of FcR Recycling

In addition to providing evidence for FcR recycling, these experiments suggest that internalized receptors may return to the plasma membrane directly from endosomes. The pH dissociation profile of 2.4G2 Fab from the FcR (half-maximal at pH 4.7–4.8) is such that extensive traffic of the complex through lysosomes (pH 4.6–4.8) should result in the discharge of a significant percentage of the antibody from the receptor. However, Fab was neither accumulated intracellularly nor released into the medium, but was returned to the plasma membrane still bound to receptor. The subcellular fractionation experiments supported this possibility. Even after prolonged incubations at 37°C, most intracellular Fab sedimented in a low density fraction that was relatively devoid of acid hydrolase activity. Taken together, these findings suggest that the recycling of internalized Fab-FcR complexes occurs primarily from a population of prelysosomal endosomes, whose internal pH may not be low enough to cause disruption of the complex. Assuming no other factors are involved, the data of Figs. 5 and 6 suggest that significant dissociation of Fab from FcR should not occur at pH's above 5.2–5.3. Although endosomes have been shown to contain an ATP-

driven proton pump and an acidic interior (10, 25), these considerations suggest that endosome pH in J774 cells is not lower than 5.2.

A lysosomal intermediate in the recycling pathway is nevertheless conceivable, at least assuming that the Fab-FcR complex resides in lysosomes only briefly so as to minimize its dissociation. However, at pH 4.6, the disruption of the complex is quite rapid ($t_{1/2} < 1.5$ min), similar to the rate of dissociation of mannose-terminal glycoproteins from their receptor at pH 6 (26). In contrast to 2.4G2 Fab, mannosyl glycoproteins are efficiently accumulated by cultured macrophages. It is clear from the density gradients, however, that at least a small fraction of the internalized Fab does reach high density secondary lysosomes, accounting for the slow but measurable rate of ¹²⁵I-Fab degradation. Conceivably, the delivery of small amounts of Fab to lysosomes reflects an inefficient dissociation of Fab from FcR in endosomes, as would be expected at pH's <6 (Fig. 5). Like other markers of endosome content (e.g., horseradish peroxidase, ligands dissociated from receptors), most of this Fab would subsequently be transferred to lysosomes (1, 16).

Other Approaches to Membrane Recycling

In the past several years, evidence supporting membrane and receptor recycling has been obtained using a variety of direct and indirect approaches (see reference 1 for review). Several laboratories have previously used antibodies against defined (15, 27) or undefined (28) plasma membrane proteins to study this phenomenon. These studies have used bivalent (or multivalent) conventional antisera and have labeled second antibodies as detection reagents. Although this work has yielded results consistent with recycling, the processes observed have been much less efficient and/or considerably slower than that described here.

Certain receptor-bound ligands have also been used in a manner analogous to the present paper. Notably, the work of Tietze et al. (26) and Karin and Mintz (29) have shown that internalized ligand can be rapidly released into the medium following reculture at 37°C using mannose-terminal glycoproteins (in NH₄Cl-treated macrophages) and transferrin (in teratocarcinoma cells). The transferrin data are of particular interest since, like 2.4G2 Fab, internalized transferrin is localized to and presumably "recycles" from a low density endosome compartment (30).

Endosomes and Membrane Recycling

In considering a possible prelysosomal pathway for membrane recycling, it is interesting to note that the low pH dependent behavior of a number of ligands for receptor-mediated endocytosis usually does not require acidities as low as that attained in lysosomes. For example, the discharge of ligands from receptors usually occurs at pH's >5.5: mannose phosphate-containing glycoproteins and α_2 -macroglobulin in fibroblasts (14, 31), mannose-terminating glycoproteins in macrophages (25), asialoglycoproteins in hepatocytes (32), and iron discharge from transferrin (33). A similar situation applies to the low pH (pH <6.0) dependent penetration of Semliki Forest virus, which is known to occur in prelysosomal endosomes (34). Direct measurements that use fluorescein fluorescence have suggested that endosomes have an internal pH of 5–5.5 (25, 30). Given the possible heterogeneity of the endosome compartment, these pH estimates are somewhat

difficult to interpret. Nevertheless, it is clear that in many cases, free receptors should be generated in endosomes soon after internalization. By analogy with the results presented here, receptors could then return directly to the plasma membrane to be re-used for subsequent rounds of ligand uptake. Accordingly, endosomes are likely to play a central role in controlling membrane recycling by providing an acidic but nonhydrolytic compartment in which ligands can be removed from receptors and from which membrane recycling can occur.

Significantly, the inclusion of FcR in this proposed pathway seems to be subject to modulation. As described in the accompanying paper (4), interaction of FcR with multivalent IgG-containing immune complexes, in contrast to monovalent Fab, results in the rapid delivery of ligand and receptor from endosomes to lysosomes where both are degraded. Since IgG complexes do not dissociate from FcR at acidic pH, they should remain bound to receptor after reaching endosomes. Unlike 2.4G2 Fab, the multivalent nature of the IgG-complexes should also cause the cross-linking of adjacent FcR. This raises the possibility that the state of receptor aggregation on the endosome membrane determines whether a particular receptor is recycled to the cell surface or is removed from the recycling pathway and transferred to lysosomes. However, IgG complexes also differ from 2.4G2 Fab in that they are physiological ligands that bind FcR via the Fc domains of intact IgG molecules. Whether the binding of Fc domains may itself influence the intracellular transport of FcR is not yet clear. Nevertheless, the results of the experiments using a second antibody to cross-link Fab-FcR complexes suggest that simple aggregation may be sufficient to cause transport from endosomes to lysosomes (Fig. 8). Although the second antibody induced transfer of Fab to lysosomes was still far less efficient than in the case of multivalent immune complexes (P. Ukkonen, A. Helenius, and I. Mellman, unpublished results; 4), it must be noted that under the conditions used, most of the second antibody may have bound only monovalently to Fab-FcR. We are currently preparing covalently linked polymers of 2.4G2 Fab to provide more conclusive data about the role of receptor clustering in controlling the intracellular transport of FcR.

The authors thank George Palade, Ari Helenius, Mark Marsh, Ralph Steinman, and Cindy Galloway for helpful discussions, Katherine Potter for assistance with the manuscript, and Pam Ossorio for photographic work.

These studies were supported by grants from the National Institutes of Health (GM 20975) and the Swebilius Fund for Cancer Research. Pentti Ukkonen is supported by a Research Fellowship from the National Institutes of Health Fogarty International Center (F05-TW03314) and by grants from the Finnish Cultural Foundation and the Virustautien tutkimuksen kannatusyhdistys. Ira Mellman is a recipient of a Junior Faculty Research Award (JFRA-26) from the American Cancer Society.

Received for publication 9 May 1983, and in revised form 11 October 1983.

REFERENCES

- Steinman, R. M., I. S. Mellman, W. A. Muller, and Z. A. Cohn. 1983. Endocytosis and the recycling of plasma membrane. *J. Cell Biol.* 96:1-27.
- Mellman, I. S., and J. C. Unkeless. 1980. Purification of a functional mouse Fc receptor through the use of a monoclonal antibody. *J. Exp. Med.* 152:1048-1069.
- Unkeless, J. C., H. B. Fleit, and I. Mellman. 1981. Structural aspects and heterogeneity of immunoglobulin Fc receptors. *Adv. Immunol.* 31:247-270.
- Mellman, I., and H. Plutner. 1984. Internalization and fate of macrophage Fc receptors bound to polyvalent immune complexes. *J. Cell Biol.* 98:1170-1177.
- Mellman, I. S., H. Plutner, R. M. Steinman, J. C. Unkeless, and Z. A. Cohn. 1983. Internalization and degradation of macrophage Fc receptors during receptor-mediated endocytosis. *J. Cell Biol.* 96:887-895.
- Ralph, P., J. Pritchard, and M. Cohn. 1975. Reticulum cell sarcoma. An effector cell in antibody-dependent cell-mediated immunity. *J. Immunol.* 114:898-905.
- Unkeless, J. C. 1979. Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. *J. Exp. Med.* 150:580-596.
- Porter, R. R. 1959. The hydrolysis of rabbit α -globulin and antibodies with crystalline papain. *Biochem. J.* 73:119-131.
- Fraker, P. J., and J. C. Spreck, Jr. 1978. Protein and cell membrane iodinations with a sparingly soluble chloramide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. *Biochem. Biophys. Res. Commun.* 80:849-857.
- Galloway, C. J., G. E. Dean, M. Marsh, G. Rudnick, and I. Mellman. 1983. Acidification of macrophage and fibroblast endocytic vesicles *in vitro*. *Proc. Natl. Acad. Sci. USA.* 80:3334-3338.
- Harms, E., H. Kern, and J. A. Schneider. 1980. Human lysosomes can be purified from diploid skin fibroblasts by free-flow electrophoresis. *Proc. Natl. Acad. Sci. USA.* 77:6139-6143.
- Goldstein, J. L., and M. S. Brown. 1974. Binding and digestion of low density lipoproteins by cultured human fibroblasts: comparison of cells from a normal subject and a patient with familial hypercholesterolemia. *J. Biol. Chem.* 249:5153-5162.
- Basu, S. K., J. L. Goldstein, R. G. W. Anderson, and M. S. Brown. 1981. Monensin interrupts the recycling of low density lipoprotein receptors in human fibroblasts. *Cell.* 24:493-502.
- Gonzalez-Noriega, A., J. H. Grubb, V. Talkad, and W. S. Sly. 1980. Chloroquine inhibits lysosomal enzyme pinocytosis and enhances lysosomal enzyme secretion by impairing receptor recycling. *J. Cell Biol.* 85:839-852.
- Wilcox, D. K., R. P. Kitson, and C. C. Widnell. 1982. Inhibition of pinocytosis in rat embryo fibroblasts treated with monensin. *J. Cell Biol.* 92:859-864.
- Helenius, A., I. Mellman, D. Wall, and A. Hubbard. 1983. Endosomes. *Trends Biochem. Sci.* 8:245-250.
- Brown, M. S., R. G. W. Anderson, and J. L. Goldstein. 1983. Recycling receptors: the round-trip itinerary of migrant membrane proteins. *Cell.* 32:663-667.
- Ohkuma, S., and B. Poole. 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Natl. Acad. Sci. USA.* 75:3327-3331.
- Merion, M., and W. S. Sly. 1983. The role of intermediate vesicles in the adsorptive endocytosis and transport of ligand to lysosomes by human fibroblasts. *J. Cell Biol.* 96:644-650.
- Mellman, I. 1983. Membrane recycling during endocytosis. In *Molecular and Cellular Aspects of Lysosomes, Volume 7. Lysosomes in Biology and Pathology*. J. T. Dingle, R. Dean, and W. Sly, editors. Elsevier Biomedical Press, Amsterdam. In press.
- Muller, W. A., R. M. Steinman, and Z. A. Cohn. 1980. The membrane proteins of the vacuolar system. II. Bidirectional flow between secondary lysosomes and plasma membrane. *J. Cell Biol.* 86:304-314.
- Muller, W. A., R. M. Steinman, and Z. A. Cohn. 1983. The membrane proteins of the vacuolar system. III. Further studies on the composition and recycling of endocytic vacuole membrane in cultured macrophages. *J. Cell Biol.* 96:29-36.
- Mellman, I. S., R. M. Steinman, J. C. Unkeless, and Z. A. Cohn. 1980. Selective iodination and polypeptide composition of pinocytic vesicles. *J. Cell Biol.* 86:712-722.
- Steinman, R. M., S. E. Brodie, and Z. A. Cohn. 1976. Membrane flow during pinocytosis. A stereologic analysis. *J. Cell Biol.* 68:665-687.
- Tycko, B., and F. R. Maxfield. 1982. Rapid acidification of endocytic vesicles containing α_2 -macroglobulin. *Cell.* 28:643-651.
- Tietze, C., P. Schlesinger, and P. Stahl. 1982. Mannose-specific endocytosis receptor of alveolar macrophages: demonstration of two functionally distinct intracellular pools of receptor and their roles in receptor recycling. *J. Cell Biol.* 92:417-424.
- Louvard, D. 1980. Apical membrane aminopeptidase appears at site of cell-cell contact in cultured kidney epithelial cells. *Proc. Natl. Acad. Sci. USA.* 77:4132-4136.
- Schneider, Y.-J., P. Tulkens, C. deDuve, and A. Trouet. 1979. Fate of plasma membrane during endocytosis. II. Evidence for recycling (shuttle) of plasma membrane constituents. *J. Cell Biol.* 82:466-474.
- Karin, M., and B. Mintz. 1981. Receptor-mediated endocytosis of transferrin in developmentally totipotent mouse teratocarcinoma stem cells. *J. Biol. Chem.* 256:3245-3252.
- van Renswoude, J., K. R. Bridges, J. B. Harford, and R. D. Klausner. 1982. Receptor-mediated endocytosis of transferrin and the uptake of Fe in K562 cells: identification of a non-lysosomal acidic compartment. *Proc. Natl. Acad. Sci. USA.* 79:6186-6190.
- Maxfield, F. R. 1983. Weak bases and ionophores rapidly and reversibly raise the pH of endocytic vesicles in cultured mouse fibroblasts. *J. Cell Biol.* 95:676-681.
- Hudain, P. L., W. E. Pricer, Jr., G. Ashwell, R. J. Stockert, and A. G. Morell. 1974. The isolation and properties of a rabbit liver binding protein specific for asialoglycoproteins. *J. Biol. Chem.* 249:5536-5543.
- Dantry-Varsat, A., A. Ciechanover, and H. F. Lodish. 1983. pH and the recycling of transferrin during receptor-mediated endocytosis. *Proc. Natl. Acad. Sci. USA.* 80:2258-2262.
- Marsh, M., E. Bolzau, and A. Helenius. 1983. Penetration of Semliki Forest virus from acidic prelysosomal vacuoles. *Cell.* 32:931-940.