

Fc Receptor-mediated Binding and Endocytosis by Human Mononuclear Phagocytes: Monomeric IgG Is Not Endocytosed by U937 Cells and Monocytes

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ABSTRACT Fc receptor-mediated endocytosis of monomeric IgG1 by human mononuclear phagocytes was evaluated under conditions where aggregated IgG and insulin readily undergo receptor-mediated internalization. U937 cells or normal human peripheral blood monocytes were incubated at 37°C in the absence of free radioligand after having first bound ¹²⁵I-IgG1 at 0°C. To determine the amount of cell-associated IgG1 internalized after varying periods of 37°C incubation, surface-bound IgG1 was removed by sequential exposure of cells at 0°C to a nonspecific proteinase for 1 h and to acetic acid at pH 3.2 for 3 min. The failure to develop a proteinase- and acid-resistant fraction, similar to that seen over time at 37°C in parallel experiments with ¹²⁵I-insulin and ¹²⁵I-aggregated IgG, and the lack of degradation of the IgG1 released into the medium from the same cells over time show that these cells do not endocytose and degrade monomeric IgG by an Fc receptor-specific mechanism and suggest that constitutive recycling without degradation is unlikely to be occurring. These data fulfill one prediction of the hypothesis that receptor-receptor interaction triggers Fc receptor-mediated endocytosis.

The molecular mechanism whereby IgG immune complexes bound to Fc receptors (FcRs)¹ initiate the process of endocytosis is largely unknown. One hypothesis advanced to explain this event is that endocytosis is triggered by receptor-receptor interaction resulting from cross-linking of two or more FcRs by multivalent ligands. The testable predictions of this hypothesis, as it relates to endocytosis by mononuclear phagocytes, are (a) that monomeric ligand is not endocytosed, whereas (b) dimeric or small oligomeric ligand complexes are endocytosed. Although endocytosis of oligomeric IgG complexes has been documented (21, 35), the fate of FcR-bound monomeric IgG remains unclear. Two recent studies suggest that monomeric IgG is internalized by an FcR-mediated mechanism in murine and human mononuclear phagocytes (21, 35). This finding has been used to argue that a "conveyor belt" model (6) may best describe ligand internalization and that receptor cross-linking may not be necessary as a trigger

for endocytosis (35). However, the design of experiments to test monomeric IgG endocytosis is complicated by several factors. First, the FcR may recycle under certain circumstances (28, 29). Additionally, monomeric IgG may become aggregated under conditions that favor endocytosis (3, 7, 17) and may thereby be endocytosed as an oligomeric complex. Another problem is high nonspecific uptake of IgG (2) causing insensitivity of the assay. Thus, small amounts of FcR-mediated endocytosis and degradation by phagocytic cells incubated at 37°C with high concentrations of monomeric radioligand are difficult to ascribe with certainty to monomeric IgG uptake (21, 35).

We have attempted to evaluate endocytosis of monomeric IgG ligand by a different approach, which circumvents the difficulties mentioned above. Our method eliminates all unbound radioligand and thus minimizes nonspecific uptake, rebinding of dissociated ligand, and generation of aggregated ¹²⁵I-IgG during the 37°C period of incubation. This method offers the additional advantage of enabling detection of recycling of monomeric ligand, and by inference, recycling of ligand-occupied receptor, between the plasma membrane and an intracellular compartment. Under these conditions, we

¹ Abbreviations used in this paper: AggHGG, aggregated human IgG; BBD buffer, balanced salt solution with 1 mg/ml bovine serum albumin and 100 U/ml deoxyribonuclease; FcR, Fc receptor; TCA, trichloroacetic acid.

have found no evidence for FcR-mediated endocytosis, degradation, or recycling of monomeric IgG.

MATERIALS AND METHODS

Buffers: Phosphate-buffered saline (PBS) at pH 7.0 contained 20 mM phosphate and 146 mM NaCl. Balanced salt solution was prepared at pH 7.2 (31). For washing cells at 0°C and for suspension of cells during incubation with ligand at 0°C, buffer BBD was prepared; balanced salt solution was supplemented with 1 mg/ml fraction V bovine serum albumin (Sigma Chemical Co., St Louis, MO) and 100 U/ml deoxyribonuclease I (DN-25, Sigma Chemical Co.) to inhibit cell clumping and titrated to pH 7.2. When cells were resuspended in buffer at 37°C to evaluate endocytosis, BBD was further buffered by the addition of 10 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid (Sigma Chemical Co.), 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid (Sigma Chemical Co.), 15 mM HEPES (Gibco Laboratories, Grand Island, NY), and 2 mM Na₂HPO₃ and was titrated to pH 7.4 before use (buffered medium) (12, 38). Cell lysis buffer consisted of 1% Nonidet P-40 (Sigma Chemical Co.), 2 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.), and 500 kallikrein inactivator units/ml of Aprotinin (Mobay Chemical Corp, New York) in PBS.

Cells: U937 cells (42), originally obtained from Dr. Peter Ralph, Sloan-Kettering Institute for Cancer Research, Rye, NY, were maintained in RPMI 1640 medium (Gibco Laboratories) supplemented with 10% fetal calf serum (Sterile Systems, Logan, UT), 100 U/ml penicillin (Gibco Laboratories), and 100 µg/ml streptomycin (Gibco Laboratories). Cells in the late logarithmic growth phase were chilled to 0°C and washed three times in BBD before use. Viability, assessed by trypan blue exclusion, was consistently >95%.

Human peripheral blood monocytes were purified from the sediment of single donor plateletpheresis packs from normal blood bank donors. These sediment packs, representing the residual cells after centrifugation of plateletpheresis packs to remove platelet-rich plasma, contained the mononuclear cell fraction from the equivalent of 6–10 U of blood (37). Each pack typically contained 2–4 × 10⁹ mononuclear cells of which 10–30% were monocytes. To purify the mononuclear cell fraction, the cells, originally in 15–30 ml of acid citrate dextrose-anticoagulated serum, were first centrifuged at 180 g to remove residual platelets and then were centrifuged through an Isohpaque-Ficoll step gradient to remove erythrocytes and granulocytes (37). The mononuclear cells obtained were washed, then resuspended at 4 × 10⁶ cells/ml, in RPMI 1640 medium supplemented with 20% fetal calf serum. 90 ml of this cell suspension were then added to each of four 150-cm² fibronectin-coated tissue culture flasks (Costar Data Packaging, Cambridge, MA) prepared by the method of Freundlich et al. (11) using acid citrate dextrose-anticoagulated human plasma as a source of fibronectin. These flasks were then incubated at 37°C for 40 min to allow monocytes to adhere (and to allow dissociation of *in vivo* bound IgG), washed with medium 199 (Gibco Laboratories) supplemented with 26.8 mM NaHCO₃, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml fungizone (all reagents from Gibco Laboratories) to remove lymphocytes, and treated for 15 min with a 1:1 mixture of medium 199 supplemented with 20% fetal calf serum (Sterile Systems), and 10 mM EDTA (Fisher Scientific Co., Fair Lawn, NJ) in PBS to release purified monocytes as described (11). Cells so obtained were chilled, washed three times with BBD, and resuspended at 20 × 10⁶ cells/ml in the same buffer. Viability, as assessed by trypan blue exclusion, was consistently >95%. Monocyte purity, as estimated by cell size analysis on a Coulter model ZBI particle volume analyzer with channelizer (Coulter Electronics Inc., Hialeah, FL) was consistently between 85% and 95%.

Proteins: Human IgG1 myeloma proteins, generously provided by Dr. George Abraham, University of Rochester, were purified by ion-exchange chromatography (2) and were radioiodinated by the chloramine T method to a specific activity of 5–10 µCi/µg (16). The radioiodinated proteins were stored at 4°C in the presence of 1 mg/ml bovine serum albumin (Sigma Chemical Co.) and 0.02% Na₂S₂O₃. Porcine insulin (Eli Lilly and Co., Indianapolis, IN), ¹²⁵I-labeled to 75 µCi/µg by the chloramine T method as described (25), was a generous gift of Dr. J. N. Livingston, University of Rochester. Human IgG was purified from Cohn fraction II of pooled human serum (Miles Laboratories, Naperville, IL) by ion-exchange chromatography on diethylaminoethyl cellulose (Whatman Chemical Separation, Inc., Clifton, NJ) (11). ¹²⁵I-labeled, heat-aggregated complexes of human IgG (AggHGG) prepared as described (19) were provided for us by Dr. David Knutson, University of Rochester. The complexes contained 70–100 IgG molecules each when analyzed by ultracentrifugation through sucrose-density gradients and were labeled to a specific activity of 0.1 µCi/µg.

Endocytosis: In this paper the term binding is used to refer to ligand that associates with the cell at 0°C. The general term uptake refers to ligand that has been allowed to interact with the cell at 37°C and therefore represents both bound and endocytosed ligand. Specific or receptor-mediated binding is defined as radioligand binding or uptake (total uptake) minus binding or uptake

measured in the presence of a large excess (200-fold) of unlabeled ligand (nonspecific uptake).

The uptake of radioligand by cells was evaluated by two distinct protocols, one measuring uptake in the presence of unbound or free radioligand, and the other measuring uptake of cell-bound ligand by cells washed free of unbound radioligand. In the first protocol, cells at 10⁷/ml in buffered medium were incubated at 37°C with various radioiodinated ligands (12 nM ¹²⁵I-human IgG1, 4 µg/ml ¹²⁵I-AggHGG, and 3 nM ¹²⁵I-insulin). Duplicate cell suspensions containing, in addition to radioligand, a large excess of uniodinated ligand (2 µM human IgG1, 2 µM human IgG1, and 0.6 µM insulin, respectively) were used to measure nonspecific uptake. Periodically, portions of the cell suspensions were removed to determine ligand internalization by the procedure described below (stripping procedure).

In the second protocol, used to measure radioligand uptake in the absence of free radioligand, cells at 10⁷/ml in BBD were incubated at 0°C with various radioligands for varying times (3 nM ¹²⁵I-human IgG1 for 16 h, 4 µg/ml ¹²⁵I-AggHGG for 16 h, and 3 nM ¹²⁵I-insulin for 5 h) until equilibrium was reached. A duplicate set of cell suspensions was maintained for the measurement of nonspecific binding, exactly as described above. The cells were subsequently separated from unbound radioligand by washing three times at 4°C in BBD and were resuspended at 10⁷/ml in buffered medium containing a large excess of unlabeled ligand (500 nM human IgG or insulin). The cell suspensions were incubated at 37°C and portions were periodically evaluated for ligand internalization by the following procedure.

Stripping Procedure: The cells were stripped of surface bound ligand by sequential exposure to proteinase and acetic acid to evaluate internalization of ligand by cells at 37°C. 250 µl of cell suspension were rapidly chilled to 0°C by adding 1 ml of cold BBD and placing the suspension in an ice-water bath. The cells were subsequently kept at 0°C throughout all procedures. The cell suspensions were centrifuged for 3 min at 180 g, and the cell pellets were resuspended in 250 µl of balanced salt solution containing 5 mg/ml of a nonspecific proteinase obtained from *Streptomyces griseus* (Proteinase type XIV, Sigma Chemical Co.) and 100 U/ml deoxyribonuclease I (Sigma Chemical Co.). After 1 h at 0°C, the cells were washed three times in BBD, were resuspended in 250 µl of 0.025 M acetic acid and 0.3 M NaCl (pH 3.2), and were incubated for 3 min at 0°C. Cell-associated radioligand was separated from free either by centrifuging triplicate 50-µl samples of cells through a phthalate oil mixture as described (2) or by washing the cell pellet of the entire 250-µl sample once in 5 ml of BBD. The amount of cell pellet-associated radioligand was measured by gamma scintilligraphy in a Packard model 3002 scintillation spectrophotometer (Packard Instruments Co., Inc., Downers Grove, IL). The results of the two washing methods were no different. The means and standard deviations of replicate samples were calculated. All results were corrected for nonspecific uptake by the following formula: $S = T - NS$, where S is specific, T is total, and NS is nonspecific. "Unstripped" uptake of radioligand, which included both the radioligand that could be stripped off the surface and the radioligand that had been internalized, was measured by using duplicate samples of cells subjected to a protocol identical to the above except for substitution of BBD for both proteinase- and acid-containing solutions.

Evaluation of Ligand Degradation: The cell pellets of stripped and unstripped cells were lysed for 30 min at 0°C in 1% Nonidet P-40 cell lysis buffer. Insoluble material was removed from the lysates by centrifugation at 1,800 g. The trichloroacetic acid (TCA) solubility of the radioligand in the lysates and of cell-associated radioligand released into the medium was measured by incubating portions of lysates or medium with 12.5% TCA (Sigma Chemical Co.) and 5 mg/ml bovine serum albumin (Sigma Chemical Co.) for 20 min at 0°C and comparing the radioactivity in the precipitates with the radioactivity of the entire sample according to the following formula: % TCA precipitate = $\{ \text{cpm}(\text{pellet}) / \text{cpm}(\text{pellet}) + \text{cpm}(\text{supernatant}) \} \times 100$. Samples of medium containing radioligand released from cells at 37°C and samples of the Nonidet P-40 lysates of stripped and unstripped cells were dissolved in SDS PAGE sample buffer at a final concentration of 2% SDS (Bio-Rad Laboratories, Richmond, CA), 10% glycerol and 5% 2-mercaptoethanol (Sigma Chemical Co.), and 0.001% bromphenol blue (Eastman Chemicals, Rochester, NY). These were then immersed in a boiling water bath for 2 min, and subjected to electrophoresis on an SDS-10% polyacrylamide slab gel according to the method of Laemmli (22). The gels, after being stained, destained, and dried, were exposed to prefogged Xomat AR x-ray film (Eastman Kodak Co., Rochester, NY) and sandwiched between two intensifying screens (Lightning Plus Chronex, Dupont Instruments, Wilmington, DE) at -70°C (23). Estimates of relative band intensity of autoradiograms were made by integration of data obtained by laser densitometry.

RESULTS

Determination of the rate of extent of FcR-mediated endocytosis of monomeric IgG bound to the surface of human

mononuclear phagocytes required a method for distinguishing between internalized ligand and surface-bound ligand. Inasmuch as the rate of dissociation of IgG from these receptors is very slow at 0°C ($t_{1/2} = \sim 12$ h [1, 2] vs. $t_{1/2}$ of a few minutes for murine mononuclear phagocytes [43]), recycling or endocytosis of radioligand-occupied FcR under 37°C conditions, which allowed receptor traffic, was conveniently evaluated by rapidly chilling the cells to 0°C, washing away unbound radioligand, and stripping the surface free of bound radioligand using proteinase and acid. Internalized radioligand remained cell-associated and was readily quantified by determining the amount of radioactivity in the cell pellet after centrifugation. All data shown represent specific FcR-associated radioligand calculated by subtracting nonspecific uptake of radioligand from total uptake. Nonspecific uptake was defined as the amount found in the presence of a 200-fold excess of unlabeled ligand.

Our first experiments determined the efficiency of the stripping procedure. U937 cells, at 10×10^6 cells/ml, were incubated with 3 nM ^{125}I -IgG1 for 14 h at 0°C. Under these conditions, receptor occupancy was near 100%. Cells were then washed free of unbound ligand and stripped of surface bound IgG by sequential exposure at 0°C to proteinase at 5 mg/ml for 1 h and to acetic acid at pH 3.2 for 3 min. Cell-associated IgG was virtually all removable (98%), showing monomeric IgG to be surface-bound and confirming the absence of FcR-mediated ligand internalization at this temperature (39). As judged by microscopic inspection at $\times 450$, the cells remained morphologically intact after the stripping procedure. Cell viability, as measured by trypan blue exclusion, was 98%. Cell recovery was identical to control cells processed in parallel without exposure to proteinase and acid (85%). Stripped cells, upon return to cell culture, resumed their normal proliferative rate.

The stripping procedure was then used to evaluate receptor-mediated endocytosis of AggHGG and insulin, two ligands that should be endocytosed at 37°C by U937 cells (20, 42; also Livingston, J. N., manuscript submitted for publication). Suspensions of U937 cells at 10×10^6 cells/ml were incubated with either 0.4 $\mu\text{g}/\text{ml}$ AggHGG or 3 nM insulin at 37°C (Fig. 1). At intervals, portions were removed from the incubation

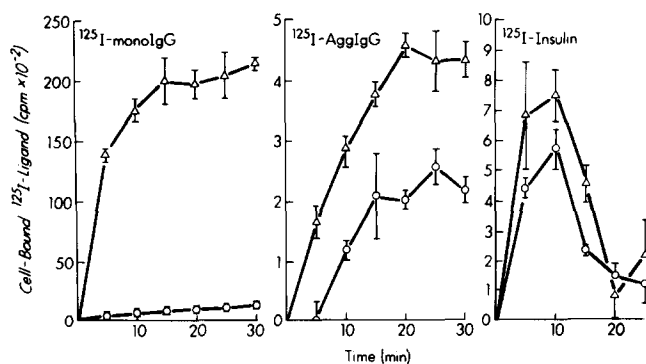


FIGURE 1 Receptor-specific uptake of radioligand by U937 cells at 37°C. Cells at $10^7/\text{ml}$ were incubated at 37°C with 11 nM ^{125}I -human IgG1, 4 $\mu\text{g}/\text{ml}$ ^{125}I -AggHGG, or 3 nM ^{125}I -insulin. Periodically, cells (0.5×10^6) were assayed for cell-bound radioligand after stripping (○) or without stripping (Δ) as described in the text. Nonspecific uptake was subtracted from total uptake to yield specific cell-bound ligand which is plotted vs. time of incubation at 37°C.

suspension and the cells were either stripped of surface-bound ligand to evaluate internalization (stripped), or were evaluated for total amount of cell-associated radioligand (unstripped). By 15 min, 50% of the cell-associated AggHGG was resistant to removal by proteinase and acetic acid, implying that the cell-associated ligand had become internalized. Similarly, 75% of cell-associated insulin was internalized by 15 min. In contrast, when U937 cells were incubated with 3 nM monomeric IgG (Fig. 1), no large influx of IgG was seen at 10 min; in fact, at 30 min only 5% of total bound IgG could not be stripped. However, for a number of reasons discussed below, it is difficult to be certain that this small quantity of unstripped ligand was strictly monomeric and was in fact endocytosed by an FcR-mediated mechanism.

We therefore modified our protocol to evaluate endocytosis of AggHGG, insulin, and monomeric IgG under conditions where minimal free radioligand was present in the medium and where our observations could be confined to the fate of those molecules surface-bound to FcR at 0°C. Under these conditions, nonspecific uptake was near zero, chances for aggregate formation were minimal, and rebinding and pinocytosis of dissociated IgG was inhibited. U937 cells were incubated with radioligand at 0°C until receptor occupancy was near 100%. Cells were then washed free of unbound radioligand and were resuspended at 37°C in the presence of an excess of unlabeled ligand to inhibit rebinding of dissociated IgG. Nonspecific binding, measured in a separate set of cells exposed to a 200-fold excess of unlabeled IgG, was $<2\%$ of total binding. At intervals, the total amount of FcR-associated radioligand was determined and the cells were stripped to evaluate the amount of internalized FcR-associated ligand. After the 0°C incubation but before warming of the cells to 37°C, nearly all of the bound ^{125}I -AggHGG (91%) and ^{125}I -insulin (97%) was removed with proteinase and acid, indicating that these ligands remained surface-bound (Fig. 2). However, after 8 min at 37°C, a significant fraction of cell-associated insulin (64%) and AggHGG (39%) could no longer be removed by the stripping procedure, implying that the ligands had moved to the interior of the cell. On the other hand, monomeric IgG bound to the surface of U937 cells at 0°C was not interiorized at 37°C (Fig. 3). Instead, the FcR-bound monomeric IgG dissociated from the cell in a log-linear fashion ($t_{1/2} = 12$ min) (2). During the entire course of the dissociation, all but a small constant amount (2% of that bound at 0°C) of FcR-bound ligand was stripped from the cells by proteinase and acid (Fig. 3). Ultracentrifugation of the ligand to deplete it of aggregated protein did not alter the result, nor did the use of another human IgG1 myeloma protein or pooled human IgG. Moreover, sampling at 1-min intervals during the first 10 min showed no evidence of a transient peak of unstrippable radioligand.

Inasmuch as the small fraction (2% of total bound) of FcR-associated monomeric radioligand that resisted stripping was present at the start of the 37°C incubation and did not increase with time, it is not likely to be a manifestation of the time- and temperature-dependent processes of endocytosis. However, if endocytosed radioligand were being degraded and returned to the medium very rapidly such that the internal pool of IgG at any one time were very small, then the stripping procedure would not have detected an increase in interiorized ligand and would have overlooked endocytosis of monomeric IgG. A further consideration is that the small fraction of FcR-associated monomeric radioligand that resisted stripping may

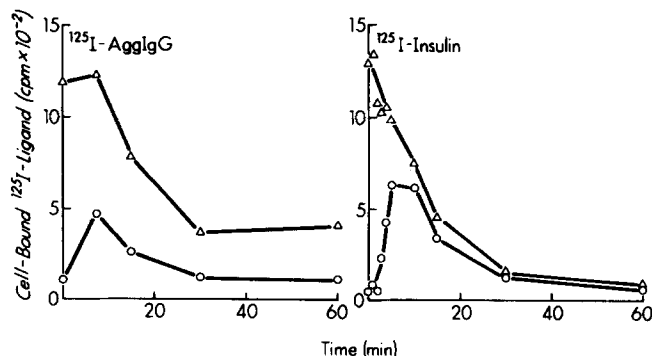


FIGURE 2 Receptor-bound radioligand remaining cell-associated at 37°C. U937 cells (10^7 /ml) were incubated with 4 μ g/ml 125 I-AggHGG for 16 h at 4°C or 3 nM 125 I-insulin for 5 h at 4°C. After the cells were washed free of unbound radioligand, a 200-fold excess of unlabeled ligand was added and the temperature was raised to 37°C. Periodically, cells (0.5×10^6) were assayed for cell-bound radioligand without stripping (Δ) or after stripping the cells free of surface-bound ligand with proteinase and acid (O). The data plotted represent specific or receptor-mediated uptake calculated by subtraction from total binding of nonspecific binding measured in the presence of a 200-fold excess of unlabeled ligand. Calculations based upon specific activity of radiolabeling indicated 42,000 125 I-IgG molecules bound/cell (≈ 70 molecules/aggregate) and 1,600 molecules 125 I-insulin bound/cell at the beginning of 37°C incubation. U937 cells bear 3,800 high-affinity insulin receptors/cell (Livingston, J. N., manuscript submitted for publication).

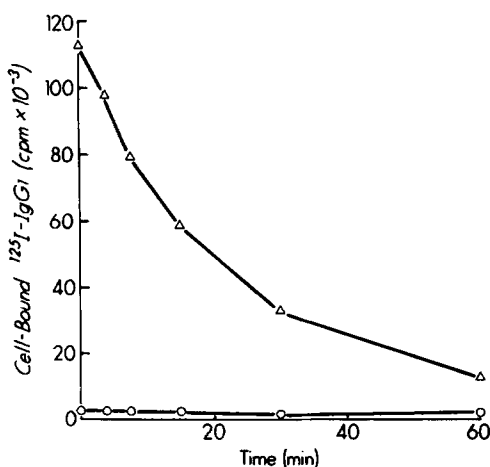


FIGURE 3 Receptor-bound 125 I-IgG1 remaining cell associated at 37°C. The experimental protocol is the same as that described in the legend to Fig. 2 except that 3 nM 125 I-IgG1 was incubated with cells for 18 h at 4°C and 2.5×10^6 cells were assayed at each timed interval. Calculations based upon specific activity of radiolabeling indicated 12,600 molecules 125 I-IgG1 bound/cell at the beginning of 37°C incubation. The results are representative of five similar experiments.

have contained a degradative pool of endocytosed IgG. These possibilities were evaluated by looking for evidence of degradation of radioligand associated with the cells or released from the cells during the 37°C incubation period of the experiment shown in Fig. 3. Radioligand solubility in trichloroacetic acid and analysis of heavy and light chain size by SDS PAGE and autoradiography were used as indicators of ligand degradation. Stripped and unstripped cells from the experiment shown in Fig. 3, were lysed in 1% Nonidet P-40 detergent.

Radioligand in the detergent lysates of unstripped cells was >97% precipitable in 12.5% TCA throughout the course of the 37°C incubation, as was the IgG released into the medium by these same cells over time. IgG from the detergent lysate of stripped cells was 80% TCA-precipitable prior to beginning the 37°C incubation and precipitability did not decrease during the course of the 37°C incubation, suggesting no ongoing degradation. Analysis of these three sources of IgG by SDS PAGE and autoradiography (Fig. 4) revealed that the IgG associated with unstripped cells over time at 37°C, and that released into the supernatant from these same cells, appeared undegraded; that is, only intact IgG heavy and light chains were seen on autoradiograms of gels run in the presence of 2-mercaptoethanol (Fig. 4, center and right panels). The radio-labeled material at the buffer front in the case of the supernatant (Fig. 4, right panel) is also present in the (far right) lane analyzing the stock 125 I-IgG1, which had not been incubated with cells, and therefore does not represent cell-mediated degradation of ligand. SDS PAGE of IgG from detergent lysates of cells that had been stripped with proteinase and acetic acid revealed (Fig. 4, left panel), in addition to heavy and light chains, the presence of two additional discrete cleavage fragments of $\sim 15,000$ and $30,000$ mol wt, which together constituted $\sim 30\%$ of the radioactivity in the lysate. Because these fragments were present before the cells were warmed to 37°C (i.e., immediately after the 0°C binding period) and because they did not increase in density upon warming the cells to 37°C, they are likely due to the proteinase stripping procedure itself rather than to cell-mediated degradative processes. In the gel that evaluated stripped cell lysate, an additional band moving slightly faster than light chain appeared at 30 and 60 min. This band represents $<0.3\%$ of the total FcR-bound ligand and thus might very well represent a degradation fragment of the $<2\%$ nonspecifically bound IgG, some of which may have become endocytosed upon warming of the cells.

Finally, experiments similar to those described above for U937 cells were performed with normal human monocytes. Monocytes incubated with AggHGG overnight at 0°C were washed free of unbound ligand, warmed to 37°C in the presence of excess cold ligand, and were periodically stripped with proteinase and acetic acid. As with the U937 cells, a large fraction (80%) of cell-associated AggHGG became proteinase- and acid-resistant by 8 min (not shown), indicating that these cells were capable of receptor-mediated internalization under our experimental conditions. However, when

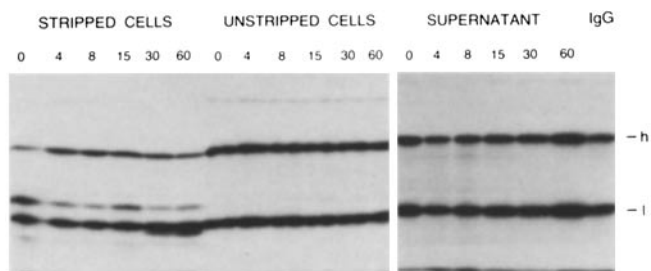


FIGURE 4 SDS PAGE autoradiogram of U937 cell-associated and dissociated 125 I-IgG1 during 37°C incubation. Detergent lysates of the stripped and unstripped cells and portions of the 37°C supernatant from an experiment like that in Fig. 3 were analyzed by SDS PAGE and autoradiography. Lanes, left to right in each set represent 0, 4, 8, 15, 30, and 60 min of incubation. The far right lane is a sample of the 125 I-IgG1 ligand with which the cells were incubated.

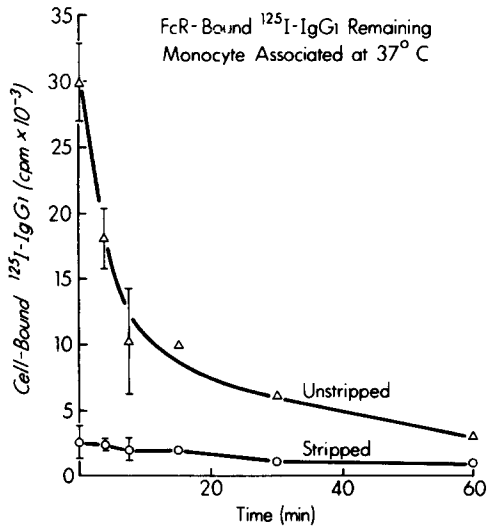


FIGURE 5 FcR-bound ^{125}I -IgG1 remaining monocyte-associated at 37°C . The protocol is the same as in Fig. 3. The means and standard deviations of three experiments are shown; only one of these three experiments was continued beyond 8 min.

monocytes were incubated with monomeric IgG using similar experimental conditions, no evidence for internalization was seen (Fig. 5). As with U937 cells, the vast majority of monocyte-bound monomeric IgG (91%) could be stripped from the cells before they were warmed from 0°C . Further, during the 37°C incubation, no subsequent increase in proteinase- and acid-resistant monomeric IgG was seen. Analysis by TCA precipitation and by SDS PAGE and autoradiography of monomeric IgG released into the medium and associated with stripped and unstripped cells, as with U937 cells, showed no evidence for ligand degradation (not shown).

DISCUSSION

Two conclusions regarding FcR-mediated endocytosis can be drawn from these studies on human mononuclear phagocytes. First, the IgG FcR does not mediate endocytosis and degradation of monomeric IgG. Second, monomeric IgG bound to the FcR is not recycled, and, by inference, the FcR does not recycle constitutively when occupied by ligand.

Prior work on the subject of FcR-mediated endocytosis of IgG by mononuclear phagocytes is confined to studies involving incubation of cells with high concentrations of monomeric radioligand for lengthy periods at 37°C . Under these conditions other workers have concluded that murine macrophage-like cells (P388D1) endocytose $\sim 15,000$ molecules/cell per 30 min (35) and that human monocyte-like cells (U937) internalize 300–3,300 molecules/cell per 3 h (21). These data (35) have served as a cornerstone of the “conveyor belt” model for endocytosis (6, 35). This hypothesis states that receptors are internalized at a fixed rate and that ligand monomers and oligomers are endocytosed at rates proportional to the time they remain bound to the cell membrane. By using a similar protocol, we too found that both U937 cells (Fig. 1) and normal human monocytes endocytose by FcR-mediated mechanisms a small amount ($\approx 5\%$ of total bound) of IgG from a pool of monomeric IgG. However, the conditions of these experiments preclude our attributing with certainty this small amount of uptake to receptor-mediated endocytosis of monomeric IgG. The problems are threefold. At 37°C in the

presence of a high concentration of radiolabeled IgG, nonspecific uptake of monomeric IgG is high (70% of total binding at 30 min) so the ratio of specific radioligand uptake to nonspecific uptake is relatively low (~ 0.4). Moreover, rebinding of FcR-dissociated IgG may elevate nonspecific pinocytosis by maintaining a high local concentration of radioligand (9). Most importantly, the temperature of incubation and the secreted products of the actively metabolizing cells may have contributed to the generation of a small percentage of aggregated ligand in the monomeric IgG (3, 7, 17). This small percentage of aggregates may have been selectively bound and endocytosed by the cells over time at 37°C . Aggregates of IgG constituting only 0.3% of the total radioligand incubated with the cells would have accounted for the result seen in Fig. 1.

We therefore devised an alternative method for evaluating monomeric IgG endocytosis avoiding the several possible artifacts of the procedure described above. This approach was possible because of the slow rate of dissociation of ligand from the FcR on these human cells (2). Cells whose FcR were occupied by monomeric IgG were washed free of unbound ligand and were then warmed to 37°C to allow endocytosis of bound ligand to proceed. Surface bound ligand was stripped from the cells with proteinase and acid while internalized radioligand remained cell-associated. Thereby, a single wave of endocytosis could be evaluated prior to complete dissociation of the bound ligand. The advantages of this approach are several. First, nonspecific binding is $<2\%$ and thus small amounts of endocytosis should be detectable. Second, in the presence of excess unlabeled ligand, reassociation of FcR-dissociated labeled ligand is inhibited. Therefore the enhancement of nonspecific IgG pinocytosis due to elevated local concentration of IgG seen in the absence of unlabeled ligand does not occur (9). Third, the opportunity for incorporation of radioligand into newly formed aggregates is minimal.

Under these alternative experimental conditions—excess unlabeled ligand but minimal radioligand in the medium—no evidence for FcR-mediated endocytosis of monomeric IgG was seen (Fig. 3). The rate of dissociation of monomeric ligand from these cells was sufficiently slow ($t_{1/2} \approx 12$ min) (Fig. 3) that ligand internalization should have been perceptible. In virtually all receptor systems evaluated, the rate of ligand endocytosis is quite rapid, $t_{1/2}$ being on the order of 3–5 min (4, 6, 8, 40, 41). The uptake of immune complexes by phagocytic cells has as well been shown to be on the order of a few min (24, 27, 35). Moreover, these same U937 cells and monocytes displayed maximum uptake of both AggHgg and insulin within 8 min (Figs. 1 and 2). Yet by 8 min neither U937 cells nor monocytes had endocytosed monomeric IgG despite the fact that 50–60% of FcR-bound IgG remained cell-bound. It is important to point out that these same experiments could not have been done with murine macrophages because the rate of ligand dissociation of IgG from FcR ($t_{1/2} \approx 2.6$ min for IgG2a) is more rapid than the generally observed rate of ligand endocytosis (10, 15, 35, 36, 43).

In addition to our failure to find direct evidence for ligand interiorization, no indication was seen of FcR-mediated degradation of monomeric IgG. Were cells endocytosing and degrading bound monomeric ligand too rapidly to be perceived as a pool of intracellular IgG, then degradation products of bound radioligand would have appeared in the incubation medium. Yet analysis of the supernatants and of the cell-associated radioligand by SDS PAGE and autoradiography (Fig. 4) and by TCA precipitation indicated that the great

majority of bound as well as dissociated radioligand remained intact during the 37°C incubation period. Other data (not shown) indicated that the monomeric IgG which dissociated from the cell was capable of rebinding to FcR, further suggesting that IgG was still intact.

The second conclusion to be drawn from these data is that receptor-bound IgG (and by inference the receptor itself) is not recycled between the plasma membrane and an intracellular pool of receptor-ligand complexes. When the cells were warmed to 37°C in the absence of free ligand, no evidence was seen for a rapid uptake of ligand (Fig. 3). Similarly, bound radioligand did not rapidly equilibrate with a perceptible intracellular pool during the first few min when the cells were incubated with free ligand at 37°C (Fig. 1). Recycling, however, would have been overlooked were the internal pool size very small (less than the quantity of unstrippable bound IgG1, 2%) and were the recycling rate very rapid ($t_{1/2} < 1$ min) compared with our earliest measurement of internalization. These conditions, however, are quite different from the internal pool size and endocytic rate seen with any receptor system thus far described (4, 6, 36, 40).

Although our data suggest that the FcR for IgG on human mononuclear phagocytes does not recycle, two lines of evidence have indicated that the murine macrophage FcR for IgG2b is capable of recycling under certain circumstances. Studies using the 2.4G2 anti-FcR antibody to precipitate the FcR from selectively radioiodinated endocytic vesicles have shown that the receptor is equally represented in the endocytic vesicle membrane and the plasma membrane (29). Further, experiments tagging the IgG2b FcR with radioiodinated Fab fragments of 2.4G2 antibody have shown that 20–25% of the tagged receptor is rapidly internalized and reexpressed ($t_{1/2} \sim 3$ min) on the cell surface (28). The finding that P388D1 cells endocytose $\sim 15,000$ IgG2a molecules/cell per 0.5 h, although not interpreted as such by the authors, may indicate recycling as well (35). Whether the disparity between our results and those of others are methodologic or represent fundamental differences in FcR behavior between species, cell types, or receptor types is unclear. One easily testable possibility is that FcR recycling is a manifestation of cells more differentiated than U937 and circulating monocytes. Cultured monocytes (18) and lymphokine-induced U937 cells (14) will be used to evaluate this idea.

Our data showing that FcR-bound monomeric IgG is not endocytosed fulfills one of the predictions of the hypothesis that receptor-receptor interaction triggers the process of FcR-mediated endocytosis. This hypothesis states that cross-linking of FcR by oligomers of IgG generates a signal that initiates the endocytic process; receptor-bound monomers of IgG cause no such cross-link or signal. Such a hypothesis has the virtue of unifying a number of different observations about receptor biology generally and about specific responses evoked by immune complexes. For example, receptor cross-linking would explain the repetitive generation of a signal that initiates movement of the leading edge of phagocyte plasma membrane around an IgG-coated particle during the (“zipper”) process of phagocytosis (13). For those soluble immune complexes too small to be taken up by a phagocytic mechanism, receptor-receptor interaction may trigger receptor-ligand endocytosis in small endocytic vesicles, perhaps in coated pits (26). Although we failed to find evidence for receptor recycling, this hypothesis would easily accommodate constitutive recycling of FcR between the plasma membrane

and an endocytic pool (5), as has been found with murine macrophages (28). FcR cross-linking by immune complexes might simply interrupt the constitutive recycling process or might initiate a superimposed response such as shunting of immune complex-bearing endosomes to lysosomes (27). Even in the absence of endocytosis, FcR cross-linking has been shown to result in membrane perturbation causing depolarization (44) and mediator release (32). Further appeal for this hypothesis comes from evidence that several diverse biological responses are triggered by receptor-receptor interaction, responses as diverse as mast cell degranulation (30) and peptide hormone action (33, 34).

The other prediction of the receptor cross-linking hypothesis has already been demonstrated (21, 35); namely, small oligomers of IgG, even dimers, stimulate endocytosis. Indeed, the rate of internalization is independent of oligomer size (Table II of reference 35). Evaluation of both the valence of the FcR and the capacity of dimeric immune complexes to stimulate endocytosis will be required to test whether FcR dimerization constitutes a sufficient signal for induction of endocytosis.

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