FATE OF PLASMA MEMBRANE DURING ENDOCYTOSIS

I. Uptake and Processing of Anti-Plasma Membrane and Control Immunoglobulins by Cultured Fibroblasts

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ABSTRACT

The uptake and processing by cultured rat embryo fibroblasts of control rabbit immunoglobulins (C IgG) or IgG directed against plasma membrane constituents (anti-PM IgG), and labeled with fluorescein (F) or with radioactive acetate (A), have been investigated by cell fractionation and immunological techniques.

Both F and A anti-PM IgGs become bound to the cell surface, by a process that is slow, but largely temperature-independent. In the presence of an excess of high-affinity antibodies, binding reaches an absolute limit which corresponds to extensive coating of the plasma membrane. The anti-PM IgGs remain attached to the membrane for at least several days, even at 37°C, with no significant transfer to lysosomes or degradation.

In contrast, C IgGs are handled very differently by the fibroblasts, and their fate is strikingly affected by the type of labeling used. AC IgG is taken up slowly, at a rate proportional to its concentration, and is subsequently broken down in what appears to be lysosomes. Part of the AC IgG also binds to the plasma membrane. FC IgG is taken up many times faster than AC IgG, though with the same strict linearity as a function of concentration. Most of the FC IgG taken up is stored in cytoplasmic granules which behave like lysosomes. For reasons that are not understood, only about half of the stored FC IgG can be broken down.

Cells exposed simultaneously to AC IgG and FC IgG, or to A anti-PM IgG and FC IgG, handle each type of IgG in its characteristic fashion.

Kinetic analysis of these results indicates that AC IgG could be taken up by fluid endocytosis, but that FC IgG must be interiorized by a selective mechanism, presumably adsorptive in nature.

That anti-PM antibodies remain stably bound to the plasma membrane and do not interfere with the uptake of FC IgG is interpreted to indicate either that two distinct membrane domains are involved in the two phenomena, or that membrane patches coated with anti-PM IgG participate in endocytosis, and are recycled back to the cell surface after delivering their contents intracellularly.
KEY WORDS: endocytosis, immunoglobulins, lysosomes, plasma membrane, recycling.

The experiments described in this paper were originally started with the aim of exploring the use of antibodies as target-specific carriers for chemotherapeutic drugs according to the general "lysosomotropic" model proposed by our group (5, 31). For this purpose, labeled purified anti-plasma membrane antibodies were offered to fibroblasts, and their fate was followed biochemically, with the help of an analytical density gradient fractionation procedure known to resolve lysosomes from other organelles in such cells (34). The results revealed, rather surprisingly, that the antibodies taken up by the cells remain stably bound to the plasma membrane for several days, with no sign of shedding or degradation, interiorization, or transfer to lysosomes. It was also found in control experiments that fluorescein conjugation modifies dramatically, both qualitatively and quantitatively, the manner in which immunoglobulins are handled by cells.

As a result of these observations, the work developed into a study of pinocytosis and of the fate of the plasma membrane in this process. The results reported in this and in the companion paper (20) provide strong support to the concept that plasma membrane fragments participating in pinocytosis return to the cell surface after the materials taken up have been delivered to lysosomes or to some other intracellular storage compartment. Preliminary reports of part of the work described have appeared elsewhere (19, 35, 37).

MATERIALS AND METHODS

Purification of Plasma Membranes and Lysosomes

A fraction enriched in plasma membranes (PM) was isolated from rat liver by the method of Song et al. (23). PM were further purified by density equilibration in a sucrose gradient after selective increase of their buoyant density by addition of digitonin (27, 28). The final preparation (PM fraction) was enriched 30- to 35-fold in 5'-nucleotidase and alkaline phosphodiesterase. Its contamination by endoplasmic reticulum, mitochondria, and lysosomes amounted altogether to <10% of the total protein, as estimated from marker enzyme determinations and the relative specific activity values given by Leighton et al. (14) for the pure cell components. The PM fraction was washed with 0.15 M NaCl and suspended in phosphate-buffered saline (PBS).

PM from cultured fibroblasts were purified in a similar way from a microsomal fraction isolated by differential centrifugation (34). The final preparation was enriched 17-fold in 5'-nucleotidase.

Lysosomes were isolated from the livers of rats treated with Triton WR-1339 (30). A soluble and an insoluble lysosomal fraction were separated by centrifugation after the lysosomes had been ruptured by dialysis against 1 mM sodium bicarbonate (29).

Preparation, Purification, Labeling and Characterization of IgG

Non-specific control IgG (C IgG) was prepared from large commercial batches of rabbit sera by chromatography on DEAE-Sephadex A-50 (22); it was then labeled with [3H]- or [14C]acetic anhydride (AC IgG) (12), or with fluorescein isothiocyanate (FC IgG) (26). The labeled IgG contained 1-2 mol of acetate or 1-3 mol of fluorescein per mol of protein.

Sera directed against liver PM were obtained from rabbits treated with PM fractions mixed with Freund's adjuvant (29). Globulins were precipitated from anti-PM antisera by half-saturated ammonium sulfate, and then labeled with [3H]acetic anhydride or with fluorescein isothiocyanate. The labeled anti-PM antibodies were then purified by immunoadsorption on PM fragments prepared by comminution in a French press and linked to Sepharose 2B by the method of Cuatrecasas (4). The antibodies were eluted with 2% formic acid, which was immediately removed by filtration of the eluate through Sephadex G-25 equilibrated with PBS. The eluted material consisted exclusively of IgG as indicated by immunoelectrophoresis, and was retained quantitatively by an excess of PM-Sepharose. Its labeling ratio was the same as that of control IgG.

IgG against liver PM was found to crossreact with the PM of fibroblasts, as shown by immunofluorescence (see below, Fig. 3 a), and by immunohistobiontion of 5'-nucleotidase. It did not bind significantly to membranes from liver mitochondria, endoplasmic reticulum or Golgi apparatus, but did react with soluble and insoluble lysosomal fractions (9). For some experiments, anti-PM IgG was freed from this crossreacting material by immunoadsorption on an excess of soluble and insoluble lysosomal fractions immobilized on Sepharose 2B (4).
About 40% of the IgG was removed in this process.

Monovalent Fab' was prepared from anti-PM IgG according to Nisonoff et al. (17), but with dithiothreitol (5 mol/mol IgG) as reducing agent.

Goat anti-R IgG was obtained from commercial antisera, and labeled by acetylation, by techniques similar to those applied to rabbit IgG.

**Cell Culture, Harvesting, and Fractionation**

Rat embryo fibroblasts were obtained and cultivated to confluence in either glass or plastic vials containing a modified Eagle's medium, supplemented with 10% calf serum, as previously described (34). The cells were then incubated for a further period of time in the same medium containing IgG preparations as described in the text. At the end of the experiments, the cells were washed three times with PBS at room temperature. They were then either dissolved in 1% Na deoxycholate adjusted to pH 11.3, and analyzed for their IgG content, or detached by a 20-min incubation at 37°C in 0.5 mM EDTA-PBS, washed in ice-cold 0.25 M sucrose-1 mM EDTA, homogenized and fractionated as described earlier (34). Isopycnic centrifugation was performed on postnuclear supernates (PNS), which contained >80% of all the constituents that were assayed. Results are presented in the form of density distribution histograms (14).

**Biochemical and Chemical Analysis**

Assay methods for most enzymes and for protein have been described previously (34). Cathepsin B was measured with α-N-benzoyl-DL-arginine 2-naphthylamide (2). The 5'-nucleotidase activity was corrected for the small but distinct contribution of nonspecific phosphatases to the hydrolysis of 5'-AMP by subtracting the result of a parallel assay run in the presence of 20 mM EDTA, which completely inhibits true 5'-nucleotidase (40).

Fluorescence was measured on samples dissolved in 1% Na deoxycholate adjusted to pH 11.3, with a Perkin Elmer 204 fluorimeter (Perkin Elmer Corp., Norwalk, Conn.) at excitation and emission wavelengths of 485 and 520 nm, respectively. Radioactivity was measured in a Packard 2425 scintillation counter (Packard Instruments Co. Inc., Downers Grove, III.) after neutralization of the samples and dispersion in Biofluor (New England Nuclear, Boston, Mass.). Separation of 3H and 14C was carried out, when necessary, with a sample oxidizer (Oxymat, Intertechnique S. A., Plaisir, France). Electrophoresis was performed on polyacrylamide gels, in the presence of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (13).

**Fluorescence Microscopy**

Nonconfluent cells growing on glass coverslips were examined in a Leitz Orthoplan Ploem type fluorescence microscope (E. Leitz GmbH, Wetzlar, German Federal Republic) and photographed on Ektachrome High Speed film (Kodak).

**Materials**

All chemicals were of analytical grade. They were purchased from E. Merck A. G., Darmstadt, German Federal Republic, Sigma Chemical Co., St. Louis, Mo., or Koch Light, Colnbrook, England. Radiolabeled chemicals were purchased from The Radiochemical Center, Amersham, England. Sephadex, Sepharose, and Protein A-Sepharose CL-4B were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden; fluorescein isothiocyanate from Laboratoires Méreux, Lyon, France; Freund's adjuvant from Difeo Laboratories, Detroit, Mich.; control goat IgG and goat antisera against rabbit serum or rabbit IgG from Miles Laboratories Ltd., Slough, England. Control rabbit serum and cell culture reagents were purchased from Laboratories Eurobio S. A., Paris, France, or Gibco-Biocult, Paisley, Scotland. All solutions were sterilized by filtration through Millipore type GS filter membranes (0.22 µm Diam) (Millipore Corp., Bedford, Mass.). Rats were of the Wistar strain and bred in a local animal house; rabbits for immunization were obtained from various outbred colonies.

**RESULTS**

**Accumulation of Anti-Plasma Membrane IgG**

Some kinetic features of the uptake of anti-PM IgG by fibroblasts are illustrated in Fig. 1. As a function of time, the reaction is progressive and surprisingly slow, taking many hours before reaching a plateau. Uptake is faster for F anti-PM IgG than for A anti-PM IgG. Lowering of the temperature from 37°C to 4°C has little effect on the rate of uptake of A anti-PM IgG, and decreases the plateau level by only 20%.

The plateau level itself is directly proportional to the IgG concentration, at least within a certain range, and the proportionality factor is fivefold greater for F than for A anti-PM IgG. At high enough IgG concentration, however, accumulation reaches an absolute limit which is the same for the two antibody preparations, and corresponds to the uptake of 10 µg of IgG per mg of cell protein. This limit presumably corresponds to saturation of all available cellular binding sites.

In contrast, the plateau that is reached at non-saturating IgG concentration reflects exhaustion of high-affinity antibodies from the medium, as shown by the results represented in the upper part of Fig. 2: preincubation of anti-PM IgG at non-
**FIGURE 1** Kinetics of accumulation of anti-PM IgG by fibroblasts. Cells (~400 μg protein) were incubated in Leighton tubes containing 2.5 ml of medium, either for different durations with 20 μg of A anti-PM/ml at 37°C (A, ▲) or 4°C (A, △), or with 10 μg of F anti-PM IgG at 37°C (B, ◯), or for 36 h at 37°C with different concentrations of F anti-PM IgG (C, ●) or A anti-PM IgG (C, ▲). At the end of the incubation, the cells were washed, dissolved in 1% Na deoxycholate, and analyzed for radioactivity or fluorescence. Mean results ± SD of three independent experiments.

**FIGURE 2** Influence of prior incubation of IgG with or without cells on its accumulation by fibroblasts. Cells (~400 μg protein) were incubated for 24 h in Leighton tubes containing 2.5 ml of medium, with 10 μg of A anti-PM IgG/ml at 37°C, or 4°C or with 100 μg of FC IgG or AC IgG/ml at 37°C. At the end of incubation, the cells were washed, dissolved in 1% Na deoxycholate, and analyzed for radioactivity or fluorescence. (A) fresh IgG-containing medium; (B) IgG-containing medium preincubated for 24 h at 37°C without cells; (C) IgG-containing medium preincubated for 24 h at 37°C with a first batch of cells, then transferred to experimental cells.
saturating concentration at 37°C in the presence of cells, but not in their absence, resulted in a marked reduction of the amount of labeled antibodies taken up by a second batch of cells, either at 37°C or at 4°C. From the slopes of the lines of Fig. 1C and from the experimental conditions (≈400 μg of cell protein in 2.5 ml of medium), it can be calculated that these high-affinity antibodies represent 2% and 10% of the total IgG in the A and F anti-PM IgG preparations, respectively. Since both preparations originated from the same antiserum, it appears that more high-affinity antibodies were lost upon labeling or upon subsequent purification from the A than from the F preparation.

The association of anti-PM IgG with the cells is remarkably stable. Cells incubated with either A or F anti-PM IgG for 24 h did not lose labeled material to an appreciable extent when reincubated in fresh medium for up to 6 d. The labeled material could be detached from the cells by sonication and exposure to 2% formic acid. It behaved like intact IgG upon gel filtration (Sephadex G-150), SDS-polyacrylamide gel electrophoresis, and reaction with goat anti-R IgG immobilized on Protein A-Sepharose CL-4B.

**Subcellular Distribution of Anti-Plasma Membrane IgG**

When fibroblasts that had accumulated F anti-PM IgG were examined by fluorescence microscopy, the fluorescence was seen associated mainly with the cellular membrane, delineating the cell periphery, with formation of occasional patches (Fig. 3a). This pattern persisted for at least 36 h, without evidence of significant capping. In contrast, cells exposed to nonspecific FC IgG showed intense granular staining of the cytoplasm (Fig. 3b).

As shown in Fig. 4, cell-associated A anti-PM IgG accompanies closely the plasma membrane marker 5’-nucleotidase upon density gradient fractionation. Similar results were obtained with F anti-PM IgG and with Fab' fragments prepared from A anti-PM IgG, and also with IgG raised against purified plasma membranes from fibroblasts. Such distributions were, however, found only if the anti-PM IgG's had been freed of antibodies cross-reacting with lysosomal antigens. If this precaution was not taken, the distribution was bimodal, with a major peak accompanying 5’-nucleotidase, and a smaller one occurring in the high

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**Figure 3** Fluorescence microscopy of fibroblasts. (a) Cells incubated for 24 h at 37°C with F anti-PM IgG (50 μg/ml). (b) Cells incubated for 24 h at 37°C with FC IgG (200 μg/ml).
density region of the gradient where the lysosomes are located. This second peak probably reflects association of cross-reacting antibodies with the lysosomes, since we have found before that anti-lysosome antibodies have a special tropism for these particles (19, 32, 33, 35).

**Uptake and Processing of Control IgG**

When control IgG was substituted for anti-PM IgG in the culture medium, accumulation of label by the fibroblasts followed superficially similar kinetics, progressing towards a plateau which was reached after some 15–30 h (Fig. 5). As with anti-PM IgG, most of the accumulated labeled material, when extracted from the cells, behaved like intact IgG in several test systems, including SDS electrophoresis, gel filtration, and reaction with goat anti-R IgG immobilized on Protein A-Sepharose CL-4B.

These similarities are, however, purely incidental, and hide major differences. First, as shown in Fig. 5, the plateau value is strictly proportional to IgG concentration up to the highest concentration used. There is no sign of saturation of the cells, and the intracellular IgG content may (with FC IgG) exceed more than five times the maximum level of 10 μg per mg of cell protein observed with anti-PM IgG.

Reaching a plateau does not either reflect exhaustion of the medium in a species of rapidly cleared IgG or in some necessary factor, as indicated by the results of Fig. 2. It is also very unlikely that accumulation levels off because the cells cease to take up IgG, since doubling the IgG concentration after attainment of the plateau is followed by an immediate new wave of IgG uptake indistinguishable from the first one (Fig. 5 D).

Most likely therefore, net accumulation stops because uptake is compensated by breakdown. Indeed, the results of Fig. 6 show that stored IgG is rapidly unloaded, mostly in the form of low molecular weight breakdown products, from cells transferred to IgG-free medium. As will be pointed out in the Discussion, the observed rates of breakdown are of the right order of magnitude to account for the leveling off of the accumulation curves.

The nature of the label has a remarkable influence on the amount of IgG accumulated, which was much greater with FC IgG than with AC IgG. Experiments with doubly labeled FAC IgG have shown that this difference is due to an authentic enhancement of IgG accumulation by fluorescein conjugation, although the possibility that acetylation may, in addition, partly depress IgG accumulation cannot be entirely excluded on the basis of our results. The effect of fluorescein varied from one batch of FC IgG to another and seemed to depend critically on the number, and perhaps the location, of the lysines substituted. The observed
**Figure 5** Kinetics of accumulation of C IgG by fibroblasts. Cells (~400 μg protein) were incubated at 37°C in Leighton tubes containing 2.5 ml of medium, either for 36 h with different concentrations of AC IgG (A) or FC IgG (B), or for different durations with 100 μg of AC IgG per ml (C), with 100 μg of FC IgG/ml (D, □) or with 50 μg of FC IgG/ml (D, ▲), or with 50 μg of FC IgG/ml and doubling of this concentration after 24 h (D, △). At the end of incubation, the cells were washed, dissolved in 1% Na deoxycholate and analyzed for radioactivity or fluorescence. Mean results ± SD of three independent experiments, except for D.

Plateau levels (expressed in volume of medium cleared) were of the order of 200 μl per mg of cell protein in all experiments reported in the present paper. But values as low as one-third this value have been observed with other batches of FC IgG and even lower ones with an occasional batch of FAC IgG. In contrast, clearance of AC IgG was very reproducible, at ~8 μl per mg of cell protein.

It is clear from Figs. 5 and 6 that the main effect of fluorescein must be on the rate of uptake of the IgG. But there are also some differences in processing. When exposed to IgG-free medium, cells that have accumulated AC IgG release part of it immediately in essentially undegraded form, and break down the remainder (Fig. 6 A). In contrast (Fig. 6 B), cells that have stored FC IgG release only a small fraction of it in intact form, roughly equivalent in absolute value to the amount of intact AC IgG released. But they seem able to degrade only about half their stored IgG. About 50% of the labeled material remaining in the cells after processing had stopped was found to behave like intact IgG upon Sephadex G-100 chromatography.

In Fig. 7 are shown some results obtained on cells exposed to C IgG for 1 h at 4°C. At low IgG concentration, binding was proportional to concentration and not very different for the two preparations, corresponding to the clearance of 4 and 6 μl of medium per mg of cell protein for AC IgG and FC IgG, respectively. At IgG concentrations above 100 μg/ml, the two preparations diverged. Whereas binding of FC IgG continued to increase with increasing concentration, up to an apparent plateau of 3 μg per mg of cell protein, that of AC IgG leveled off to a much lower plateau, of the order of 0.56 μg per mg of cell protein. The significance of these results is, however, puzzling since the amount of AC IgG bound by the cells at 4°C in the presence of low IgG concentrations was ten times higher than the amount taken up in the same time at 37°C.
Control IgG and UO1E of nUO

**FIGURE 6** Release of label from C IgG-loaded fibroblasts upon transfer to fresh medium. Cells (~2 mg protein) were incubated for 24 h at 37°C in Falcon flasks containing 250 µg of either AC IgG (A) or FC IgG (B) per ml, then washed three times with PBS and reincubated in 1 ml of fresh medium. Cells and medium were analyzed after various lengths of time. O, label remaining in cells; •, total label in medium; △, labeled material of low molecular weight (Sephadex G-100) in medium. Mean results ± SD of three independent experiments.

**FIGURE 7** Low temperature binding of C IgG by fibroblasts. Cells (~1 mg protein) were incubated for 1 h at 4°C in either Falcon flasks or Petri dishes containing various concentrations of AC IgG (A) or FC IgG (B), then washed three times with PBS at 0°C, dissolved in 1% Na deoxycholate, and analyzed for radioactivity or fluorescence. Mean results ± SD of three independent experiments.

**Subcellular Distribution of Control IgG**

As illustrated in Fig. 8, cell-associated AC IgG showed a bimodal distribution suggestive of a dual localization of the IgG, partly with plasma membranes (5'-nucleotidase) and partly with lysosomes (cathepsin D and N-acetyl-β-glucosaminidase). This hypothesis, assuming biochemical homogeneity of the two sites, is expressed mathematically by the following equation, applicable to each individual fraction (6):

\[ 100c = ax + by, \]

in which \( a \), \( b \), and \( c \) are the fraction contents (in...
% of total) in 5'-nucleotidase, acid hydrolase and IgG, respectively; $x$ and $y$ are the percentages of the total labeled IgG that can be assigned to plasma membranes and lysosomes, respectively.

Applying eq. 1 to all the fractions shown in Fig. 8, we have calculated the best fitting values of $x$ and $y$ by a method of least squares (3). According to this calculation, 54% of the label distributes like 5'-nucleotidase and 38% like the lysosomal marker; 8% cannot be assigned. The distribution calculated in this manner is shown by dotted lines in Fig. 8. The fit is satisfactory, except for the presence of some soluble IgG, probably released by the experimental manipulations.

In Fig. 9 are shown the distribution patterns recorded for cell-associated FC IgG after various times of exposure to the IgG. After 30 min, the pattern resembled that of AC IgG. Later, however, it shifted progressively to an essentially unimodal distribution mimicking closely that of lysosomal hydrolases. Cells at this stage, when examined in the fluorescence microscope, showed numerous brightly lit cytoplasmic granules (Fig. 3 b). When the cells were offered doubly-labeled FAC IgG, both markers showed the typical lysosome-like distribution of FC IgG.

**Independent Uptake of Different IgGs**

In Table I are listed some results showing that AC IgG or A anti-PM IgG had no effect on the accumulation of FC IgG. Conversely, presence of the latter did not seem to affect the accumulation of AC IgG or A anti-PM IgG. We did not investigate the effect of F anti-PM IgG on the uptake of the other IgGs. There is, however, an indication that cells exposed to this antibody may be unable to take up FC IgG. At least, as shown by the plateau in Fig. 1 C, they did not take up the low-affinity antibodies present in the medium, when exposed to an excess of F anti-PM IgG.

As shown in Fig. 10, when cells exposed to a mixture of A anti-PM IgG and FC IgG were fractionated, each type of IgG went to its characteristic location, with little evidence of interaction. Whether the slight anomalous bulge of 5'-nucleotidase in the high density region of the gradient observed in this experiment was significant or accidental is not known. It will be noted that A anti-PM IgG showed the same anomaly.

**Accessibility of Cell-Associated IgG**

To obtain a direct estimate of the amount of rabbit IgG bound to the cell surface, we have used goat anti-R IgG as probe. Results are shown in Table II. Under the conditions of these experiments, cells that had not been incubated first with a rabbit IgG preparation took up only very small amounts (0.06 µg per mg of cell protein) of goat
Isopycnic centrifugation of PNS from fibroblasts incubated at 37°C for 30 min, 1 and 3 h, with 250 µg of FC IgG/ml, or for 24 h with 100 µg of FC IgG/ml. The cell contents in IgG were 1.05, 1.76, 3.56, and 21.3 µg/mg protein at the different times. Recoveries ranged from 82 to 117%.

Table 1

Independent Accumulation of FC IgG and of AC or A anti-PM IgG by Rat Fibroblasts

|          | AC IgG µg/ml | µg/mg cell protein | A anti-PM IgG µg/ml | µg/mg cell protein | FC IgG µg/ml | µg/mg cell protein |
|----------|--------------|--------------------|---------------------|--------------------|--------------|--------------------|
| 10       | 0.08 ± 0.01  | —                  | —                   | —                  | 100          | 18.7 ± 2.4         |
| 20       | 0.17 ± 0.01  | —                  | —                   | —                  | 100          | 19.2 ± 1.8         |
| 40       | 0.33 ± 0.02  | —                  | —                   | —                  | 100          | 17.0 ± 2.1         |
| —        | —            | 1.98 ± 0.02        | —                   | —                  | 100          | 18.3 ± 0.4         |
| —        | —            | 4.05 ± 0.03        | —                   | —                  | 100          | 18.9 ± 0.5         |
| —        | —            | 8.06 ± 0.01        | —                   | —                  | 100          | 17.9 ± 1.7         |

Fibroblasts were incubated for 36 h at 37°C in the presence of the IgG mixtures shown. Accumulation levels are given as means of three determinations ± SD.

anti-R IgG. Values shown in Table II have been corrected for this amount, and may be taken to represent antibody reacting with surface-bound rabbit IgG.

As shown in Table II, cells that had been incubated for 24 h with F anti-PM IgG bound as many as 4.76 molecules of goat anti-R IgG per molecule of rabbit IgG stored. Unfortunately, the anti-PM IgG preparation used in this experiment had not been freed from antibodies cross-reacting with...
Fibroblasts incubated at 37°C with rabbit IgG under conditions shown were washed three times with PBS, and reincubated for 2 h at 37°C with goat A anti-R IgG (100 µg/ml) in culture medium.

* Corrected for nonspecific fixation (0.06 µg per mg of cell protein).

† Estimated on the assumption that one molecule of exposed rabbit IgG binds 7 molecules of goat anti-R IgG (see text).

§ This preparation had not been freed of antibodies cross-reacting with lysosomal antigens, and showed a bimodal distribution, with two-thirds accompanying 5'-nucleotidase, and most of the remainder lysosomal hydrolases.

lysosomes. It showed a bimodal distribution upon fractionation, with two-thirds of the IgG accompanying 5'-nucleotidase, and the remainder following the lysosomal markers. In all probability, only the amount of IgG associated with 5'-nucleotidase was exposed on the cell surface, from which we conclude that each molecule of exposed rabbit IgG bound 7 molecules of goat anti-R IgG. This factor, which seems plausible for an IgG-IgG reaction (11), was used to estimate the amounts of exposed C IgG listed in the last column of Table II. As will be noticed, cells that had taken up AC

**TABLE II**

*Accessibility of Rabbit IgG Accumulated by Fibroblasts to Anti-Rabbit IgG Antibodies*

| Type       | µg/ml | h | µg/mg cell protein | µg/mg cell protein* | Goat IgG | Ratio | Accessible rabbit IgG | µg/mg cell protein | %   |
|------------|-------|---|--------------------|---------------------|----------|-------|------------------------|--------------------|-----|
| F anti-PM§ | 2.5   | 24| 1.33               | 6.31                | 4.76     | 0.90  | 68                     |                    |     |
| AC         | 100   | 24| 0.75               | 2.64                | 3.52     | 0.38  | 50                     |                    |     |
| FC         | 100   | 0.5| 0.45               | 1.41                | 3.14     | 0.20  | 45                     |                    |     |
|            | 1     | 1 | 1.26               | 4.88                | 3.17     | 0.21  | 17                     |                    |     |
|            | 2     | 2 | 2.27               | 1.68                | 0.74     | 0.24  | 11                     |                    |     |
|            | 4     | 4 | 4.34               | 1.62                | 0.37     | 0.23  | 5                      |                    |     |
|            | 6     | 6 | 6.49               | 1.54                | 0.24     | 0.22  | 3                      |                    |     |
|            | 24    | 24| 22.5               | 1.82                | 0.08     | 0.26  | 1                      |                    |     |

Fibroblasts incubated at 37°C with rabbit IgG under conditions shown were washed three times with PBS, and reincubated for 2 h at 37°C with goat A anti-R IgG (100 µg/ml) in culture medium.

* Corrected for nonspecific fixation (0.06 µg per mg of cell protein).

† Estimated on the assumption that one molecule of exposed rabbit IgG binds 7 molecules of goat anti-R IgG (see text).

§ This preparation had not been freed of antibodies cross-reacting with lysosomal antigens, and showed a bimodal distribution, with two-thirds accompanying 5'-nucleotidase, and most of the remainder lysosomal hydrolases.
IgG also bound considerable amounts of goat antibody, indicating accessibility of about half the stored AC IgG. In contrast, cells incubated for 24 h with FC IgG actually bound less goat antibody than did the cells incubated with AC IgG, even though their total IgG content was 30-fold higher. It is noteworthy that the absolute amount of FC IgG exposed on the surface of these cells remained essentially constant throughout the whole accumulation period. Consequently, at early times it represents a substantial part of the cell-associated IgG in agreement with the results of cell fractionation (Fig. 9).

**DISCUSSION**

**Numerical Calculations**

In the calculations referred to below, we have used the following cellular dimensions, obtained in previous studies of rat embryo fibroblasts (34, 36, 39; Van Hoof and Baudhuin, personal communication): 1 mg of cell protein corresponds to \(~2.8 \times 10^6\) cells, with a total volume of 5 \(\mu\)L, and a surface area of plasma membrane of \(~56\) cm

By comparison, Steinman et al. (24) determined that 1 mg of fibroblast-like L cell protein contains \(~2.2 \times 10^6\) cells, which occupy a volume of 3.9 \(\mu\)L and have a surface membrane area of 46 cm

For kinetic evaluations, we will assume that IgG is taken up by pinocytosis, according to the general formula (5, 10):

\[
\frac{dQ}{dt} = v \left(1 + \frac{\theta R}{K + C}\right)C,
\]

in which \(Q\) is the amount taken up, in \(\mu\)g per mg of cell protein; \(t\) is the time, in h; \(v\) is the pinocytic rate, in cm

\(R\) is the density of binding sites on the membrane, in \(\mu\)g/cm

\(C\) is the IgG concentration, in \(\mu\)g/cm

\(K\) is the IgG concentration giving half-saturation of the binding sites.

We will assume further that degradation of stored IgG is a first-order process, of time-constant \(k\) (in h

Then net uptake is given by:

\[
\frac{dQ}{dt} = v \left(1 + \frac{\theta R}{K + C}\right)C - kQ,
\]

which, all other variables remaining constant, integrates as a function of time:

\[
Q_t = Q_s \left(1 - e^{-kt}\right),
\]

in which:

\[
Q_s = \frac{v}{k} \left(1 + \frac{\theta R}{K + C}\right)C,
\]

is the IgG content of the cells in the steady state.

**Binding of Anti-Plasma Membrane Antibodies**

Results obtained with anti-PM IgGs were complicated to some extent by the occurrence, in our preparations, of antibodies cross-reacting with lysosomal constituents. When such antibodies were present, and only then, part of the labeled IgGs taken up by the cells was found to accumulate in the dense region of the gradient where the lysosomes are concentrated. The "lysosomotropic" character of anti-lysosome antibodies has been observed before (19, 32, 33, 35) and will be discussed in a forthcoming publication. It will not be considered further here.

Anti-PM IgG's freed of anti-lysosome antibodies simply combine with antigens present on the cell membrane, and remain attached to the latter for at least several days, with no appreciable evidence of interiorization, transfer to lysosomes or digestion. This view is supported by the following facts: (a) Cells incubated with F anti-PM IgG showed fluorescence only on their periphery (Fig. 3 a); this pattern remained unchanged for at least 36 h, without evidence of capping, engulfment, or accumulation in cytoplasmic granules. (b) Cells took up almost as much A anti-PM IgG at 4°C as they did at 37°C (Fig. 1 A). (c) Cells that had accumulated anti-PM IgG did not release it, whether by shedding or breakdown, when transferred to IgG-free medium. The IgG extracted from the cells behaved like intact IgG. (d) Upon density gradient centrifugation, the distribution pattern of the cell-associated anti-PM IgG paralleled that of the plasma membrane marker 5'-nucleotidase (Fig. 4); this pattern remained essentially unchanged for at least 6 d after transfer of the cells to IgG-free medium. (e) Cells that had accumulated rabbit anti-PM IgG readily took up a goat anti-(rabbit IgG) antibody, with the entirely plausible stoichiometry of seven molecules of anti-R antibody per molecule of rabbit IgG accompa-
Fate of Plasma Membrane during Endocytosis

The conditions under which such phenomena have been observed are, however, very different from those used in our work (for a review, see reference 16). With fibroblasts, capping has been seen only in transformed cells (7) but not to our knowledge in untransformed cells. In earlier experiments, the same antibody preparation has been offered to HTC hepatoma cells, with similar results: the antibody showed a close association with plasma membrane markers, stable for at least 36 h (15).

Uptake and Processing of Control IgG

In first approximation, accumulation (Fig. 5), and degradation (Fig. 6) of C IgG agree with the model of eq. 4, with k values of the order of 0.1 h⁻¹ for the two types of IgG's, and Q₁ values 20-25 times greater for FC IgG than for AC IgG. But there are complications.

In the case of AC IgG, it is obvious that two distinct cellular compartments participate in its storage, most probably represented by the plasma membrane and by lysosomes. Association of part of the stored IgG with lysosomes is supported by cell fractionation experiments (Fig. 8) and is consistent with the rapid breakdown of interiorized IgG (Fig. 6 A). Binding of the remainder of the AC IgG to the plasma membrane is evidenced by its behavior upon cell fractionation (Fig. 8), accessibility to anti-R IgG (Table II), immediate unloading as intact IgG upon transfer of the cells to fresh medium (Fig. 6 A), and binding at 4°C (Fig. 7 A). It so happens that the amounts of AC IgG we estimate to be membrane-bound at 37°C correspond exactly to the amounts measured at 4°C: 0.4 (Fig. 8 and Table II) and 0.6 (Fig. 6 A) µg per mg of cell protein at IgG concentrations of 100 and 250 µg/ml, respectively. The significance of this coincidence is, however, questionable since the cells need at least 10-15 h at 37°C to reach the degree of IgG binding they achieve in 1 h at 4°C. This temperature effect is puzzling, especially since it was not observed with A anti-PM IgG (Fig. 1 A).

Whatever the explanation, our results make it clear that the accumulation of AC IgG, as shown in Fig. 5 C, is probably a dual process, consisting partly of progressive attachment to the plasma membrane, possibly leading to an equilibrium situation, and partly of interiorization, transfer to lysosomes and breakdown, resulting in a steady state situation. According to this interpretation, the accumulation curve of Fig. 5 C is the sum of...
the attachment of low density lipoprotein (LDL) on the occasion is puzzling phenomenon. As will be discussed in detail below, it consists most likely of a mixture of high-affinity, low-affinity and non-degradable, but saturable, pool of unknown nature and uncertain cellular location.

In addition, there is a small, rapidly equilibrated pool of surface-bound FC IgG, evidenced by accessibility to goat anti-R IgG (Table II), and by the occurrence of a significant plasma membrane-like component in the subcellular distribution pattern at early times (Fig. 9). The estimates we obtain from these two types of results indicate that at 37°C the cells bind the content of ~2.4 μl of medium per mg of cell protein, at both FC IgG concentrations of 100 and of 250 μg/ml. Results obtained at 4°C (Fig. 7 B) confirm the linearity of the process but give a proportionality constant of 6.4 μl per mg of cell protein. Apparently, binding of FC IgG to the membrane is tighter at 4°C than at 37°C. A similar observation has been made for the attachment of low density lipoprotein (LDL) to its receptor in human fibroblasts (8).

The binding of both types of C IgG is itself a puzzling phenomenon. As will be discussed in greater detail below, it consists most likely of nonspecific adsorption to low-affinity binding sites, from which, as shown by Fig. 6, the IgG molecules seem to detach readily upon addition of fresh medium to washed cells. Yet, the binding resists repeated washing with PBS, homogenization and fractionation in sucrose solutions, and even exposure to medium containing an excess of anti-R antibodies. As will be shown in the subsequent paper (20), horseradish peroxidase (HRP) behaves like C IgG in several of these respects.

**Kinetic Analysis**

The most striking characteristic of C IgG uptake is its perfect linearity with concentration, a feature which is generally considered the hallmark of fluid endocytosis (5, 10, 21, 25). In the present case, however, the two IgGs are taken up at widely different rates, even when present together in the medium (Table I). Simple fluid endocytosis cannot account for this observation. There must be a selective mechanism, discriminating either in favor of FC IgG or against AC IgG. To analyze the nature of this mechanism, we have tried to fit our results to eq. 4. Due to the complications discussed above, there is some uncertainty as to the exact values to be adopted for Q, and for k.

Fortunately, whether we adopt one or the other set of values, the resulting clearance rates Q, k/C are very similar for AC IgG: 0.72 and 0.64 μl per mg of cell protein per h. They are more divergent for FC IgG, but not excessively so, especially in regard to the greater variability of the results obtained with this type of IgG: 14 and 24 μl per mg of cell protein per h. For the following analysis, we will adopt the means of the above estimates, i.e., 0.68 and 19 μl per mg of cell protein per h for AC IgG and FC IgG, respectively, as providing us with at least a correct order of magnitude.

In principle, there are two ways in which the very different rates of uptake of the two IgGs can be reconciled with theory. We can assume that FC IgG is taken up by fluid endocytosis and that the concomitant uptake of AC IgG is restricted, for instance, by a barrier interposed between the medium and the cells. Or we can assume that the uptake of FC IgG is selective and occurs by adsorptive endocytosis, or by some other process of...
unknown nature, while AC IgG is taken in by simple fluid endocytosis, or possibly by mixed endocytosis but with a considerably less favorable adsorptive component.

The first interpretation is particularly attractive for the following reasons: (a) It fits with the perfect linearity of the kinetics, a feature which can be reconciled with adsorptive endocytosis only by assuming a very low binding affinity (K much larger than 250 µg/ml, our highest value for C). (b) It agrees with our observations indicating that a barrier of the type postulated does indeed exist and that it is displaced more easily by F than by A IgG's. (c) It is not inconsistent with our observation that the cells bind comparable amounts of FC IgG and AC IgG on their surface, a fact which is very difficult to reconcile with the hypothesis of selective adsorptive endocytosis of FC IgG.

Arguing against the hypothesis of fluid endocytosis is the magnitude of the clearance rate of FC IgG, which is two orders of magnitude higher than the value measured by Steinman et al. (24, 25) on various types of fibroblasts, using HRP as substrate. Even our estimate of AC IgG clearance appears high in comparison with the results of these authors. In the subsequent paper (20), we will describe some results with HRP which point to a lower rate of fluid pinocytosis, of the order of 0.3 µl per mg of cell protein per h. This value agrees with the measurements made by Steinman et al. (25) on confluent fibroblast cultures. Thus, it appears that even AC IgG could be taken up by a partly selective process, whereas the uptake of FC IgG must be highly selective.

Adopting the hypothesis of adsorptive pinocytosis for the uptake of FC IgG, we may check for internal consistency by replacing the various terms in eq. 5 by their experimentally estimated value:

\[ v = 6.8 \times 10^{-4} \text{ cm}^3/\text{mg cell protein/h}, \]

(an upper estimate, based on AC IgG uptake),

\[ \theta = 2 \times 10^6 \text{ cm}^{-1}, \]

(from reference 24 for L cells),

\[ \frac{R}{K + C} = 0.0024 = 4.3 \times 10^{-5} \text{ cm}^{-1} \]

(from FC IgG binding, Table II). From which we calculate a clearance rate for FC IgG:

\[ \frac{Q}{C} = 6.53 \mu l/\text{mg cell protein/h}. \]

There is a threefold discrepancy with the experimental value of 19. If we adopt for \( v \) the more plausible value of 0.3 µl per mg of cell protein per h found with HRP (20), the discrepancy is even greater, 6.6-fold.

In principle, we could reconcile experiment with theory by assuming that \( \theta \) has been underestimated by Steinman et al. (24). This is possible, since very small pinocytic vesicles with a high surface to volume ratio could escape detection in the kind of morphometric analysis used by these authors. The \( \theta \) value needed to satisfy the data is, however, very high: 6.3 \times 10^5 cm\(^{-1}\), or even 14.5 \times 10^5 cm\(^{-1}\) if we adopt the lower \( v \) value. This corresponds to almost impossibly small values for the weighted average diameter of pinocytic vesicles \((2/\theta)\); 32, or even 14 nm. It also means that the cells interiorize almost eight times their surface area per hour, an enormous value.

The alternative is to assume that the density of FC IgG on the membrane is higher than our estimate, either because our determination of membrane-bound IgG is incorrect, or because the bound IgG is selectively crowded on the membrane patches involved in endocytosis. This hypothesis, however, seems almost incompatible with the remarkable linearity of the kinetics, which can be reconciled with the hyperbolic form of eq. 5 only by assuming a large enough value of \( K \). Since there is no visible deviation from linearity up to a concentration of 250 µg/ml, we must take it that \( K \) can hardly be lower than 2,000 µg/ml (1.25 \times 10^{-5} M). Combining this value with our own estimate of bound FC IgG, we obtain a value of \( R \) of the order of 0.09 µg/cm\(^2\), corresponding to a surface binding of 5 µg IgG per mg of cell protein. Compared with the experimental value of 3 µg per mg of cell protein observed at 4°C (Fig. 7 B), and with the maximal binding of 6.7 µg per mg of cell protein estimated for anti-PM IgG, this value gives a perfectly plausible order of magnitude, leaving little scope for the three to sevenfold increase in occupancy needed to satisfy our results. Either we must assume almost impossibly high \( R \) values, with available areas of 90, or even 40, nm\(^2\)/IgG molecule; or we must decrease \( K \) to a point where the uptake vs. concentration curve should show a deviation from linearity of at least 20%, which could not have escaped detection.

In conclusion, to fit our results on FC IgG uptake to the theory of adsorptive endocytosis, we have to stretch available parameters almost beyond acceptable limits. In addition, we are still left
with the problem of explaining why AC IgG, which binds to the membrane at 1.6 times the density of FC IgG, is taken up many times more slowly.

For these reasons, we suspect strongly that the mechanism of FC IgG uptake may be more complex than the model on which eq. 2 is based, involving some sort of fluorescein-induced clustering of IgG molecules on the surface of pinocytic invaginations. The nature of such a mechanism and its strictly linear response to concentration are matters of pure conjecture at the present time, although some analogy with capping comes to mind. One is reminded also of the clustering of LDL receptors in “coated pits” (1).

Membrane Phenomena

A very puzzling finding is the almost entire lack of interaction between the uptake of A anti-PM IgG and that of FC IgG (Table I, Fig. 10). It means, for one thing, that coating a major part of the membrane with tightly bound antibody does not interfere with whatever binding or clustering of FC IgG is involved in its uptake, or with the various processes of invagination and membrane fusion that determine interiorization and intracellular delivery of the IgG. The only hint of a possible disturbance is shown by the shoulder of plasma membrane markers in the lysosome region in Fig. 10, which could, however, be fortuitous.

Equally remarkable is the fact that the anti-PM IgG remains attached to the membrane for days on end, in cells which in the accomplishment of their pinocytic activity would seem to interiorize hourly between one and eight times their surface area, depending on the values of v and θ adopted.

Both types of observation would seem to suggest that the membrane patches involved in endocytosis somehow exclude the surface antigens that bind the anti-PM antibodies. How this could happen with the extensive antibody coverage to which our cells are subjected is, however, difficult to visualize.

An alternative possibility is that antibody-coated membrane areas do participate in endocytic uptake, and are continuously recycled, or shuttled, back to the cell surface. As pointed out by Steinman et al. (24), the very magnitude of membrane interiorization makes the hypothesis of recycling almost mandatory. This conclusion is, however, based on the conventional model of pinocytosis. The membrane seen around pinocytic vacuoles is assumed to be derived from the plasma membrane, but without the confirmatory assurance given by a specific marker. The anti-PM antibodies used in the present work could provide such a marker. In the subsequent paper (20), we will describe some experiments in which these antibodies were used as immunological probes to test the recycling model. As will be shown, the results obtained support this model strongly, though not in an entirely unequivocal fashion.

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