FATE OF PLASMA MEMBRANE DURING ENDOCYTOSIS

II. Evidence for Recycling (Shuttle) of Plasma Membrane Constituents

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ABSTRACT

Cultured rat embryo fibroblasts were first allowed to store for 24 h fluorescein-labeled goat immunoglobulins directed against rabbit immunoglobulins (F anti-R IgG), and were subsequently exposed for 24 h to [^{3}H]acetylated rabbit immunoglobulins known to bind to the cell membrane either specifically (anti-plasma membrane IgG: A anti-PM IgG) or unspecifically (control IgG: AC IgG). As a result of an immunological interaction between the two antibodies (no effect was found if the cells had been preloaded with control goat FC IgG), a substantial portion of the stored F anti-R IgG was unloaded from its intracellular storage site, appearing in the medium in the form of soluble immune complexes with rabbit A IgG. Part of the unloaded F anti-R IgG also was recovered in association with the plasma membrane, but only when A anti-PM IgG was used. In addition, significant reverse translocation of AC IgG from plasma membrane to lysosomes or some related intracellular storage compartment was also observed. With A anti-PM IgG, this translocation was less marked and affected at the same time the plasma membrane marker 5'-nucleotidase. Cells that had stored horseradish peroxidase (HRP) simultaneously with F anti-R IgG did not unload HRP when exposed to A anti-PM IgG.

These results support strongly, though not unequivocally, the concept that plasma membrane patches interiorized by endocytosis are recycled, or shuttled, back to the cell surface. In the framework of this concept, recycling antibody-coated membrane is taken to serve as vehicle for the selective intracellular capture and extracellular discharge of immunologically bound F anti-R IgG. The alternative explanation of regurgitation triggered off by immune complexes is considered less likely in view of the lack of HRP unloading.

KEY WORDS   endocytosis · immunoglobulins · lysosomes · plasma membrane · recycling

Abbreviations used in this paper: A, acetylated ([^{3}H] or [^{14}C]); C, control = nonspecific; F, fluorescein-labeled; HRP, horseradish peroxidase; IgG, immunoglobulin G; PBS, phosphate-buffered saline (0.15M NaCl; 2.7 mM KCL; 3mM Na$_2$HPO$_4$-KH$_2$PO$_4$, pH 7.4); PM, plasma membrane; PNS, postnuclear supernate; R, rabbit IgG.

It has been shown in the preceding paper (8) that when rat embryo fibroblasts are incubated in the

presence of an excess of purified anti-plasma membrane antibodies (anti-PM IgG) labeled either with fluorescein (F) or with radioactive acetate (A), the cells become progressively coated on their surface with high-affinity antibodies, which remain attached to the membrane for at least several days, with no significant transfer to lysosomes or degradation.

In contrast, control (C) IgGs are interiorized and broken down, in a manner and in amounts that are strikingly affected by the type of labeling. With AC IgG, uptake is slow and strictly proportional to IgG concentration up to the highest concentration used (250 μg/ml), leading to a steady-state situation in which about half the stored IgG is bound to the cell membrane, probably by nonspecific adsorption, and the remainder is stored in an intracellular compartment that behaves like lysosomes upon density gradient fractionation of the homogenized cells. This interiorized IgG undergoes digestion. FC IgG likewise binds nonspecifically to the cell membrane, in amounts that are somewhat lower, at least in the steady state and at 37°C, than the amounts of AC IgG bound by the membrane at the same IgG concentration. The rate of uptake of FC IgG, however, is much faster than that of AC IgG, even though it too is strictly proportional to IgG concentration (up to 250 μg/ml). The interiorized FC IgG is stored in cytoplasmic granules indistinguishable from lysosomes, where part of it is digested at roughly the same rate as interiorized AC IgG. Part of the stored FC IgG appears to be undegradable by the cells, for reasons that are not understood. Uptake and storage of FC IgG are essentially unaffected by the simultaneous occurrence of AC IgG or A anti-PM IgG in the medium, and reciprocally.

These findings raise a number of questions, of which the two major ones are: (a) What is the mechanism that allows the highly preferential uptake of FC IgG, without selective binding to appropriate receptors on the cell surface, and at a rate strictly proportional to IgG concentration? (b) How can a cell almost entirely coated with antibodies tightly bound to surface antigens carry out the preferential uptake of FC IgG essentially unimpaired? And, especially, how can these antibodies remain stably bound to the cell surface during days on end, in spite of an apparent pinocytic activity requiring a huge rate of membrane interiorization, amounting to at least the total surface area of the plasma membrane per hour?

No entirely satisfactory answer to these questions could be proposed within the framework of accepted models of endocytosis. As discussed in the preceding paper (8), it seems very difficult to account for the uptake of FC IgG without postulating some sort of clustering process of unknown nature. Furthermore, unless this process is unique and either does not depend on membrane interiorization or is carried out by highly segregated membrane patches, the stability of the membrane-bound antibodies makes it almost mandatory to assume that the pieces of membrane used in FC IgG uptake return to the cell surface after delivering their contents intracellularly. Even if FC IgG uptake should happen not to depend on membrane interiorization, the assumption of membrane recycling (or shuttle) would still have to be made in relation to the normal pinocytic activity of the cells, unless pinocytosis is inhibited in antibody-coated cells, or it too is carried out by highly segregated membrane patches that exclude A anti-PM antibody.

It occurred to us that our findings on the uptake of labeled IgG molecules by fibroblasts offered an interesting opportunity to put the recycling model to an experimental test. Implicit in this model is the assumption that the membrane-bound IgG molecules which seem simply to remain on the cell surface actually participate in a succession of endocytic cycles, and thus periodically "see" the inside of lysosomes for a brief interval of time during the fusion events, believed to be transient, that take place between pinocytic vacuoles and lysosomes. The occurrence of such exposures could be detected if the lysosomes should happen to contain a material with which the membrane-bound antibodies are capable of interacting specifically, for instance anti-IgG antibodies. As a result of such interactions, part of the intralysosomal antibodies could become bound to the membrane and accompany it to the cell surface. At the same time, some of the membrane-bound antibodies could be stripped off and transferred to the lysosomes. Possibly even, membrane could be immobilized with the lysosomes, and its orderly return to the surface prevented.

These predictions were tested in the following way. During a first phase, the cells were allowed to take up and store fluorescein-labeled goat antibodies directed against rabbit IgG (F anti-R IgG). Then they were washed, and incubated during a second phase in the presence of purified [³H]acetylated rabbit antibodies directed against

rat liver plasma membranes (A anti-PM IgG), or of [³H]acetylated control rabbit antibodies (AC IgG) known to bind nonspecifically to the plasma membrane. At the end of this second phase, cells and medium were collected and analyzed. As a test of the immunological specificity of any observed interaction, similar experiments were carried out on cells preloaded with control goat IgGs (FC IgG).

In another series of experiments we explored the ability of cells treated in the above manner to take up and process the conventional pinocytic substrate horseradish peroxidase (HRP).

As shown by the results described in this paper, all three predictions of the recycling model were verified, thus strengthening previous conclusions. But some questions still remain unsettled. Preliminary reports of these experiments have already appeared (6, 7, 13, 14).

MATERIALS AND METHODS

Experiments were conducted essentially as described in the preceding paper (8). The goat antibody preparations were isolated from commercial serum batches (Miles Laboratories, Slough, England), labeled with fluorescein, and eventually purified by immunoadsorption, by the same methodology as were rabbit IgG.

HRP was purchased as type II from Sigma Chemical Co., St. Louis, Mo., and labeled with [¹⁴C]acetic anhydride (4), to an average of 1–2 acetyl groups per molecule. The activity of the enzyme was not affected by this treatment. It was assayed with N,N-dimethyl-p-phenylene diamine, according to Straus (12).

RESULTS

Experimental Protocols

Table I gives the main details of the design of each type of experiment. Fibroblasts grown to confluence were first incubated for 24 h in the presence of goat F IgG, at a concentration of 100 μg/ml. The goat immunoglobulins were either nonspecific (C) or directed against rabbit IgG (anti-R).

At the end of this first incubation, the cells were washed three times with PBS at room temperature, and reincubated for a further 24 h, either without or in the presence of rabbit A IgG. This was either nonspecific (C) or directed against plasma membranes. The A anti-PM IgG had been purified by immunoadsorption and freed of antibodies cross-reacting with lysosomal constituents. It was added at a concentration ranging from 7.5 to 20 μg/ml, whereas the concentration of AC IgG was 100 μg/ml. In one experiment (exp. 7), exposure to rabbit A anti-PM IgG was limited to 4 h, after which the cells were washed and reincubated in fresh medium for another 20 h.

After the second incubation, the medium was collected and set aside for analysis. The cells were washed, detached, homogenized, and fractionated by isopycnic centrifugation in a sucrose gradient. Fractions were collected and analyzed for their content in fluorescent material (goat IgG), radioactive material (rabbit IgG) and marker enzymes for plasma membranes (5′-nucleotidase) and lysosomes (N-acetyl-β-glucosaminidase).

TABLE I

Uptake and Processing of Immunoglobulins by Fibroblasts

Experiment No.	1	2	3	4	5	6	7
First incubation, h	24	24	24	24	24	24	24
Goat F IgG, type	C	anti-R	C	C	anti-R	anti-R	anti-R
μg/ml	100	100	100	100	100	100	100
μg/mg cell protein*	8.88	7.02	9.16	8.30	6.43	5.90	6.95
Second incubation, h	24	24	24	24	24	24	4 + 20§
Rabbit A IgG, type	—	—	C	anti-PM	C	anti-PM	anti-PM
μg/ml	—	—	100	10	100	20	7.5
μg/mg cell protein	—	—	0.72	2.03	0.68	4.09	1.48
Fluorescence in medium, %‡							
high Mol Wt	0	0	0	0	21	26	11
low Mol Wt	27	28	27	26	26	26	24

* Estimated from total fluorescence of cells + medium at the end of second incubation.
‡ Percentage of total fluorescence of cells + medium
§ 4 h in the presence of rabbit A anti-PM IgG, followed by 20 h in fresh medium.

The collected incubation medium was filtered through Sephadex G-150, with PBS as eluant. The fractions were analyzed for their content in fluorescent material, which came out either as a single peak, retarded about as much as free fluorescein (low mol wt) or as two peaks, one eluting with the void volume (high mol wt) and the other like fluorescein. In exp. 7, this analysis was performed on the combined 4 + 20 h media.

Uptake and Processing of Immunoglobulins

As shown in Table I, the cells accumulated an average of 8.8 μg of goat FC IgG, and 6.6 μg of F anti-R IgG, per mg of cell protein during the first incubation, and digested ~26% of the stored IgG during the second incubation in all seven experiments. These values are lower than those reported for rabbit FC IgG (8) but still fall within the range observed with these immunoglobulins.

Capture of the two types of rabbit A IgG by the cells was similar to that observed before, and uninfluenced by the previous storage of goat F IgG. Digestion of the latter was essentially unaffected by the rabbit IgG, but the cells that had stored goat F anti-R IgG suffered an additional substantial loss of fluorescence in the form of high molecular weight material, when exposed to rabbit

IgG. This phenomenon did not occur in cells preloaded with control goat FC IgG. The material released in this way preceded authentic IgG upon gel filtration, and consisted most probably of soluble immune complexes between the goat F anti-R IgG unloaded from the cells and rabbit IgG present in the medium.

The ability of A anti-PM IgG to elicit the unloading of previously stored F anti-R IgG, but not that of FC IgG, was confirmed identically in two other experiments (see below, Table II), in which it was found in addition that the presence of HRP (350 μg/ml) during either the first or the second incubation period had no detectable effect on the amounts of immunoglobulins taken up or bound by the cells, or on their processing.

Subcellular Distribution of Immunoglobulins

In Fig. 1 are shown the subcellular distribution patterns recorded in the seven experiments of Table I. In the cells reincubated in the absence of rabbit IgG (exps. 1 and 2), or in the presence of rabbit AC IgG (exps. 3 and 5), or preloaded with control goat FC IgG (exps. 1, 3, and 4), the distribution of fluorescent material tends to follow that of the lysosomal N-acetyl-β-glucosaminidase, as observed before with rabbit FC IgG. This pattern
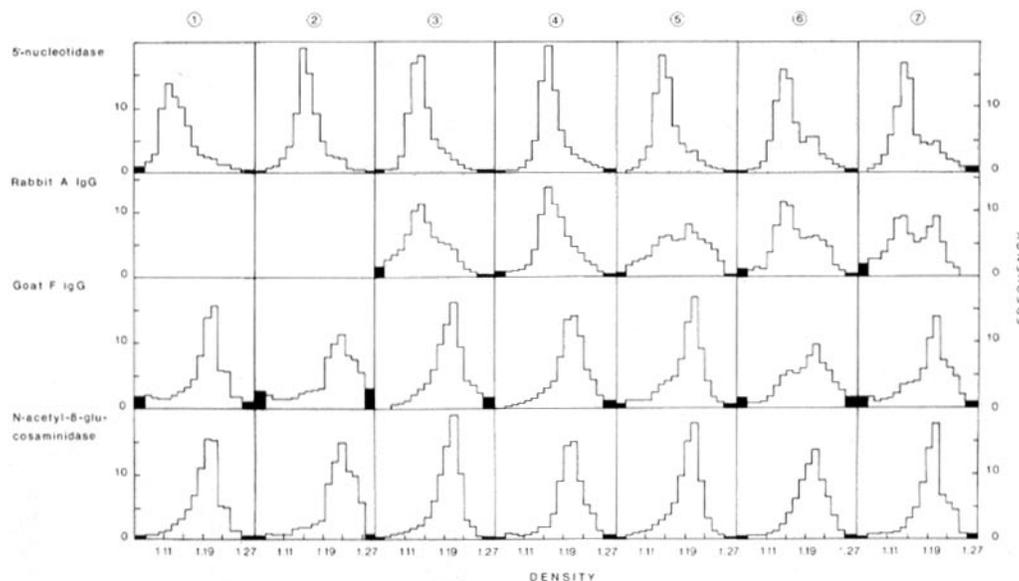

FIGURE 1   Isopycnic centrifugation of PNS from fibroblasts incubated as described in Table I. Recoveries ranged from 83 to 118%.

is, however, altered in the cells preloaded with goat F anti-R IgG and reincubated in the presence of rabbit A anti-PM IgG (exps. 6 and 7). The alteration consists in the appearance of a second peak or shoulder in the lower density region occupied by 5'-nucleotidase, and occurs without a comparable change in the distribution of the lysosomal marker.

In the cells preloaded with goat FC IgG, the distributions of rabbit AC IgG and A anti-PM IgG are similar to those observed previously without preloading. AC IgG (exp. 3) shows a major peak accompanying the plasma membrane marker 5'-nucleotidase, and a shoulder in the lysosomal region; A anti-PM IgG (exp. 4) follows 5'-nucleotidase more closely. Preloading of the cells with goat F anti-R IgG (exps. 5, 6, and 7) brings about a significant shift of radioactivity from the plasma membrane region to the lysosomal region of the gradient, causing the distribution to become clearly bimodal, even for anti-PM IgG. A similar but slighter shift affects 5'-nucleotidase in the cells exposed to A anti-PM IgG (exps. 6 and 7).

As a means of quantitating these distribution shifts, we have calculated for each experiment the proportions of labeled IgG accompanying 5'-nucleotidase and N-acetyl-β-glucosaminidase in the gradient, using the mathematical device described in the preceding paper (8). The results of these calculations are presented graphically in Figs. 2 and 3. Their validity was tested by comparison of the calculated with the experimental distributions. The fit was generally very good.

Influence of Immunoglobulins on Processing of Horseradish Peroxidase

In Table II are shown the results of two experiments in which cells were offered HRP in addition to goat F IgG during the first incubation period, and were then reincubated in the presence or absence of rabbit A anti-PM IgG. The key finding disclosed by these results is that the unloading of F anti-R IgG brought about by A anti-PM IgG is selective; it is not accompanied by a simultaneous unloading of stored HRP.

In addition, the results of Table II show some unexpected features of HRP uptake and processing by fibroblasts, which will be discussed more fully in a subsequent publication. The most striking one is the large amount of HRP released into the medium, in essentially active form, in exp. A. We have found that this is surface-bound HRP, which is not detached by washing with PBS but comes off in the presence of culture medium. In exp. B, this surface-bound HRP was removed by a single wash with medium, and only traces of enzyme were released during the subsequent 24-h incubation period. HRP binding itself we have found to be a slow process, taking many hours to reach completion. In these various respects, the
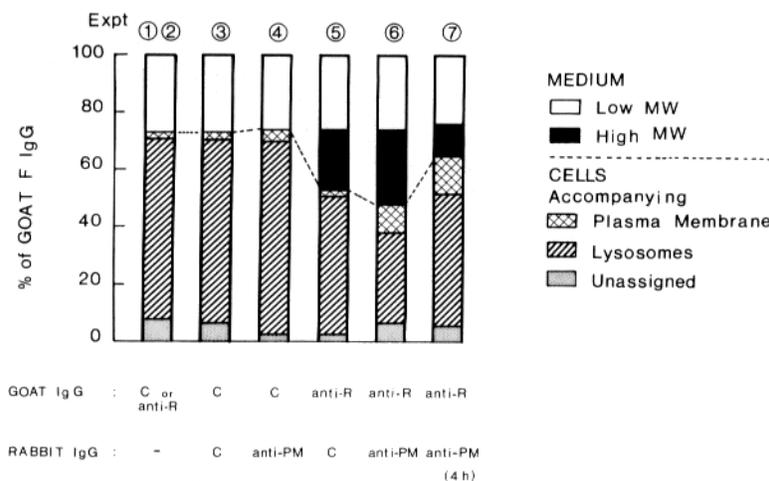
FIGURE 2   Fate of goat F IgG accumulated by fibroblasts described in Table I and Fig. 1. Proportions of intracellular IgG accompanying lysosomes and plasma membranes computed by fitting IgG distribution to those of marker enzymes N-acetyl-β-glucosaminidase and 5'-nucleotidase, as described previously (8).

Expt

100

% of RABBIT A IgG

80

60

40

20

0

③ ⑤ ④ ⑥ ⑦

Accompanying

▨ PLASMA MEMBRANE
▨ LYSOSOMES
▢ UNASSIGNED

GOAT IgG :   C   anti-R   C   anti-R   anti-R

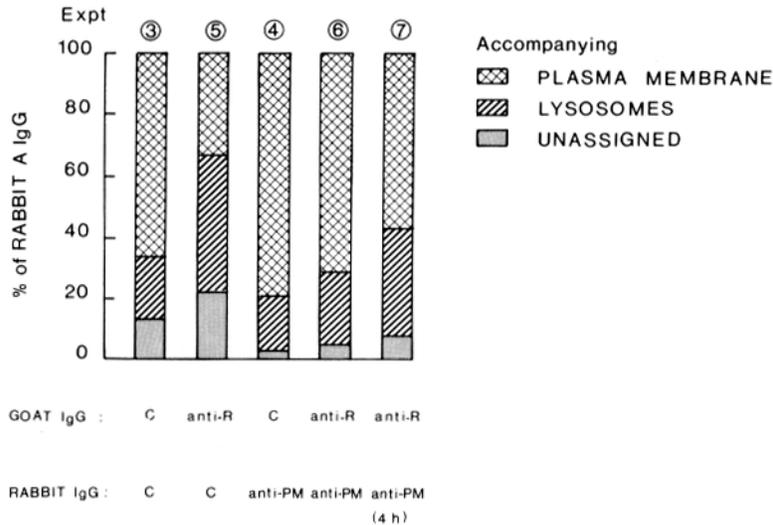RABBIT IgG :   C   C   anti-PM   anti-PM   anti-PM
(4 h)

FIGURE 3 Distribution of rabbit A IgG in fibroblasts described in Table I and Fig. 1. Presentation and calculation as for Fig. 2. Relatively large amount of unassigned IgG in exps. 3 and 5 consists mostly of soluble IgG (see Fig. 1), probably released by the experimental manipulations.

binding of HRP resembles that of AC IgG described in the preceding paper (8), except that we have no independent evidence that the binding is truly to the cells. We have verified that the cells are needed for HRP binding and that we are not dealing simply with adsorption to the walls of the culture dish, as seen by Steinman et al. (11). But adsorption to a cellular secretory product cannot at present be excluded. If the binding is to the cells themselves, it corresponds to extensive coating under the conditions of our experiments: 2×10^7 HRP molecules/cell, occupying a surface area of about 100 nm^2/molecule.

Also remarkable is the stability of the cell-associated HRP in exp. A. As shown by the ratio of enzymatic activity to radioactivity, the HRP stored by the cells suffers only 15% inactivation in 24 h, corresponding to a half-life of 102 h. Even the half-life of 26 h observed in exp. B is high, as compared to the value of 7.5 h reported by Steinman et al. (11) for confluent L cells. After inactivation of the enzyme, most of the protein-bound radioactivity remains intracellular, and there is no evidence of the release of digestion products into the medium, as occurs with F IgG.

The net rate of HRP interiorization, estimated from the radioactivity remaining with the cells after washing with medium, corresponds to a clearance rate of 0.3 μl per mg of cell protein per h. This value agrees with that observed on confluent

fibroblasts by Steinman et al. (11) but is less than half the value estimated for the uptake of AC IgG (8).

DISCUSSION

General Comments

In agreement with predictions based on the recycling hypothesis, our results demonstrate a considerable translocation of stored goat IgG, accompanied by lesser but significant changes in the cellular distribution of the rabbit IgG, in cells that are exposed to rabbit A anti-PM IgG or AC IgG after a preliminary incubation in the presence of goat F anti-R IgG. Nothing happens if control goat FC IgG is substituted for F anti-R IgG in the first incubation, making it clear that the observed effects are the result of a specific immunological interaction between the two antibodies.

These effects are best illustrated by the simplified graphs of Figs. 2 and 3. The mathematical device used to construct these graphs and its limitations have been discussed before (8). In agreement with previous results showing in a number of different cases that the proportion of cell-associated IgG accompanying 5'-nucleotidase upon fractionation was truly bound to the plasma membrane, we will assume a similar relationship in the present experiments. The location of the IgG accompanying lysosomal markers in the high density

TABLE II

Influence of Immunoglobulins on Processing of HRP by Fibroblasts

| | Experimental Conditions | | | μg HRP/mg cell protein | | | | % Fluorescence in medium | |
| | | | | Cells | | Medium | | −HRP | +HRP |
Exp.	Protocol No.*	Goat F IgG	Rabbit A IgG	Enz‡	Rad§	Enz‡	Rad§		
A (without medium	—	—	—	1.88	2.32	4.75	4.84	—	—
wash)	1	C	—	1.62	1.92	4.11	4.31	—	27
	2	anti-R	—	1.72	1.94	4.06	4.40	—	25
	4	C	anti-PM	1.65	1.90	4.03	4.29	29	27
	6	anti-R	anti-PM	1.66	1.99	3.75	4.27	49	50
B (with medium	—	—	—	1.32	2.50	0.12	0.13	—	—
wash)	1	C	—	1.41	2.63	0.14	0.16	—	26
	2	anti-R	—	1.39	2.56	0.16	0.18	—	31
	4	C	anti-PM	1.31	2.42	0.12	0.12	27	26
	6	anti-R	anti-PM	1.26	2.49	0.14	0.15	51	50

Experiments were performed as indicated in Table I, but with HRP (350 μg per ml) in medium during first incubation. At the end of first incubation, cells were washed 3 times with PBS, as usual, in exp. A, and 3 times with PBS + once with culture medium in exp. B. Concentration of goat F IgG was always 100 μg per ml; that of rabbit A anti-PM IgG was 10 μg per ml in exp. A, and 20 μg per ml in exp. B.
* See Table I for details
‡ Measured by enzyme activity
§ Measured by radioactivity

region of the gradient is more ambiguous. It undoubtedly corresponds at least partly to lysosomes, and it would be equated entirely with lysosomes were it not for the fact that only part of the stored F IgG can be degraded by the cells. Found before with rabbit F IgG, this partial resistance to intracellular digestion seems to occur also with goat F IgG, since we would expect from our previous results ($k \geq 0.07$ h^{-1}) that at least 80% of the stored F IgG should be digested in 24 h instead of the observed 25-30%, if it were entirely degradable. Due to this uncertainty, we will refer to the IgG estimated to accompany lysosomes upon fractionation as being in the "storage compartment."

The Membrane Recycling Model

According to the recycling hypothesis, the interaction between the rabbit A IgG added to the medium and the cell-associated goat F anti-R IgG is assumed to take place intracellularly, on the occasion of a fusion event believed to be transient, between a pinocytic vacuole bearing membrane-bound A IgG on its inner surface and a lysosome or other storage site filled with F anti-R IgG. As a result of this interaction, some of the stored F IgG is assumed to attach to the membrane-bound A IgG and to be carried back with it to the cell surface, from which it is subsequently detached to a greater or lesser extent by the excess soluble A IgG present in the medium. Conversely, some A IgG may be stripped off from the membrane by the F anti-R IgG present in the storage compartment. Possibly also, A IgG may be immobilized by the F anti-R IgG while remaining bound to the membrane, preventing the latter from returning to the cell surface.

If this interpretation is correct, the strength of binding of the A IgG to the membrane should affect the final result. With the tightly bound A anti-PM IgG, we should expect stored F anti-R IgG to be transferred more efficiently to the cell surface, but to be released less efficiently into the medium, than with the loosely bound AC IgG. Conversely, the loosely bound AC IgG should be transferred more readily to the storage compartment than A anti-PM IgG, whereas membrane immobilization, as revealed by the simultaneous displacement of A IgG and of 5'-nucleotidase to the high density region of the gradient should take place only with the tightly bound A anti-PM IgG. Our observations do indeed verify all these predictions.

The model also explains the considerable degree of F anti-R IgG translocation, but with greater

binding to the plasma membrane, seen in cells that were exposed to A anti-PM IgG during only 4 h and then incubated in fresh medium for a further 20 h (exp. 7). After removal of the A anti-PM IgG from the medium, together with its associated F anti-R IgG, membrane-bound A anti-PM IgG should continue recycling, becoming progressively more charged with F anti-R IgG. This, however, would now be largely retained on the membrane, since there is no competing A anti-PM IgG in the medium to either strip off F anti-R IgG from its attachment to the membrane-bound antibody, or displace an IgG-IgG immune complex from the membrane. This is exactly what we find, ending up with more F anti-R IgG attached to the membrane than is observed upon continuous exposure of the cells to the A anti-PM IgG, even though a lower concentration of this antibody was used. Combining the results of Figs. 2 and 3 with the IgG contents given in Table I, we estimate that in exp. 7 the plasma membrane bore 1.08 molecules of goat antibody per molecule of bound rabbit antibody (or 15% of the maximal load, 8). In exp. 6, this ratio is only 0.2.

One fact, however, does not entirely fit with the recycling model, namely the ability of AC IgG to promote substantial unloading of F anti-R IgG (exp. 5). Admittedly, A anti-PM IgG (exp. 6) caused 50% more IgG unloading than did AC IgG, even though its concentration was only one-fifth that of AC IgG. On the other hand, the amount of membrane-bound A IgG was only 0.23 μg per mg of cell protein for AC IgG (exp. 5), as opposed to 2.9 for A anti-PM IgG (exp. 6). It is difficult to visualize how such a small amount of loosely bound A IgG could act as carrier with two-thirds the efficiency of more than ten times the same amount of tightly bound A anti-PM IgG. A possible explanation of this discrepancy could be that soluble immune complexes formed intracellularly between stored F anti-R IgG and interiorized AC IgG also tend to bind to the membrane and to be carried back with it to the cell surface.

The quantitative efficiency of the unloading mechanism postulated by the recycling hypothesis also deserves consideration. For this, we need an estimate of the rate of membrane interiorization, a quantity on which as discussed in the preceding paper there is considerable uncertainty. As a conservative estimate we will take 60 cm^2 per mg of cell protein (1.07 times the cell surface) per h, which is obtained from the rate of HRP uptake (v

= 0.3 μl per mg of cell protein per h) and from the average surface to volume ratio of pinocytic vesicles measured by Steinman et al. (10) ($\theta = 2 \times 10^5$ cm^{-1}). Combining this value with our estimate of membrane-bound A IgG, we find for exp. 5 that a total of $1.07 \times 0.23 \times 24 = 5.9$ μg of AC IgG per mg of cell protein has allegedly cycled through the storage compartment during the 24-h incubation period, removing 1.48 μg of F anti-R IgG per mg of cell protein in the process, or 0.25 molecules of goat antibody per molecule of membrane-bound antibody cycled. In exp. 6, this ratio calculated in the same manner is only 0.028, even though A anti-PM IgG is the hypothetical carrier. In exp. 7, if we assume, as seems reasonable, that all the F anti-R IgG found in the medium was unloaded, together with an additional third of this amount remaining membrane-bound (see exp. 6), during the 4-h period when rabbit A anti-PM IgG was present, we find ratios of 0.29 for the first 4 h of incubation, and of 0.034 for the subsequent 20 h. Since one membrane-bound rabbit IgG molecule can bind as many as seven molecules of goat anti-R IgG (8), the above ratios are certainly plausible and would appear to indicate that unloading of the intracellular storage compartment by a recycling membrane-bound ligand is not a very efficient process. In addition, accessibility of the stored F anti-R IgG to the unloading mechanism seems to decrease sharply as the degree of unloading increases.

An Alternative Model: Regurgitation

An alternative explanation of our results which does not require membrane recycling is that F anti-R IgG unloading occurs by exocytic regurgitation, a process which could be triggered off by an extracellular or juxtacellular interaction between the two antibodies concerned. Indeed, regurgitation has been described in cells exposed to immune complexes (1, 2, 15), although under conditions very different from ours. Furthermore, we know from our previous results that a small, but significant, amount of F IgG is bound to the plasma membrane of cells exposed to this form of IgG. This could conceivably suffice to initiate regurgitation, after which the process could become autocatalytic, the regurgitated F anti-R IgG reacting further with the A IgG in the medium to elicit more regurgitation.

This explanation does not readily account for the changes in the intracellular distribution of the

rabbit A IgG in the cells exposed to the immune reaction. But one could visualize that the cells take up immune complexes from the medium and transfer A IgG to their lysosomes in this form.

A strong argument against regurgitation is provided by the fact that stored HRP was not discharged from cells exposed to the immune interaction. Apparently, the unloading of F anti-R IgG is selective and cannot occur by bulk exocytic discharge, unless two distinct compartments with different unloading mechanisms are involved in the storage of F IgG and HRP. We have not observed any evidence of depletion of lysosomal enzymes from the unloading cells, but this argument is of limited value in view of the ability of fibroblasts to take up lysosomal enzymes selectively from the medium (5).

CONCLUSION

Although not entirely conclusive, our results provide a strong experimental argument in support of the concept of membrane recycling during endocytosis. That such recycling must occur has been deduced previously by Steinman et al. (10) from the high rate of membrane interiorization associated with the normal pinocytic activity of cells. Our quantitative estimates reinforce this argument, since our calculations based on different premises have led to even higher estimates of membrane interiorization rate than those of Steinman et al. (10). There is also considerable evidence on secretory cells indicating recycling in the other direction of membrane material added to the plasma membrane in the course of exocytosis (9). Most likely, the two phenomena are related, with endocytosis and exocytosis serving to close the cycle at the cell surface.

The term "recycling" should, however, not be taken too literally. It is certainly possible, as suggested by this term, that plasma membrane patches travel intracellularly by a complex and circuitous itinerary which, in addition to endocytic vacuoles, lysosomes, and other "storage compartments," may encompass also the Golgi system and secretion granules (3). But it is also possible that we are dealing with a multitude of short-distance "shuttles," rather than with a cycle, and at the limit that there is no real translocation of membrane, but only transfer of contents through a series of compartments transiently interconnected by alternate

fusion-fission events, or perhaps even permanently interconnected by narrow channels fitted with appropriate locks. Whatever the itinerary involved, our results would appear to suggest that the membrane maintains its structural integrity during the process and is recycled as patches, not in the form of dissociated constituents.

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