Fate of Plasma Membrane during Endocytosis III. Evidence for Incomplete Breakdown of Immunoglobulins in Lysosomes of Cultured Fibroblasts

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ABSTRACT Rat embryo fibroblasts, when cultured in the presence of control rabbit immunoglobulins (C IgG), doubly labeled by ^{3}H-acetylation (A) and then conjugated with fluorescein (F), take up FAC IgG continuously for at least 72 h. They return the major part of their intake back to the medium in the form of breakdown products of very low molecular weight. Gel filtration and immunological analyses of cells and medium at various times indicate that essentially all the FAC IgG molecules taken up undergo digestion of their Fc part, but that the Fab part of only about three-fourths of the molecules is degraded. The rest remains stored intracellularly in the form of F(ab')2-type fragments that slowly dissociate into Fab'-type fragments.

When FAC IgG was incubated in vitro in the presence of a hepatic lysosomal extract, complete digestion of the Fc part likewise occurred, but the Fab' part of most if not all the molecules proved resistant to breakdown, and remained as Fab'-type fragments.

Cell fractionation experiments have demonstrated that the storage compartment of the FAC IgG and of its digestion residues: (*a*) shows a density distribution pattern in a sucrose gradient identical to that of the lysosomal marker *N*-acetyl-β-glucosaminidase and clearly dissociated from that of the Golgi marker galactosyltransferase, and (*b*) accompanies the lysosomal marker in its density shift induced by exposure of the cells to chloroquine.

It is concluded that storage and processing of FAC IgG by rat fibroblasts occur in a single, digestively active compartment of lysosomal nature, and that resistance to digestion of certain Fab'-type fragments accounts largely for the inability of the lysosomal enzymes to completely digest the FAC IgG taken up. This conclusion implies that the intracellular storage compartment through which, in earlier work, plasma membrane patches were found to transit after endocytosis and before recycling to the cell surface consists of authentic lysosomes.

Previous investigations have shown that cultured rat fibroblasts avidly take in fluorescein-labeled control immunoglobulin G (FC IgG), and that IgG stored in this manner can be driven back into the medium by exposure of the cells to a plasma membrane ligand capable of binding the IgG. These findings were interpreted as supporting the occurrence of a recycling or a recycling or shuttling of membrane patches between the cell surface and the intracellular storage site of the IgG (12, 13). The implications of such a mechanism are numerous and important, and depend critically on the precise nature of the storage site. Should this site, for instance, be shown unequivocally to be lysosomes, many of our accepted concepts of the functioning of these digestive organelles would have to be revised.

Several observations do indeed argue strongly in favor of a lysosomal identification of the FC IgG storage site. When viewed in the fluorescence microscope, it appears as a sprinkling of small particles randomly spread throughout the cytoplasm, as are lysosomes in fibroblasts. Upon density equilibration in a sucrose gradient, the particle-bound IgG shows a distribution identical to that of several lysosomal marker enzymes and differing clearly from those of other markers. In

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addition, active digestion of the stored IgG was found to occur, leading after a few hours to an apparent steady-state situation where IgG uptake and breakdown seem to balance each other.

There is, however, one disturbing fact. When fibroblasts that have reached this apparent steady state after 24 h of exposure to FC IgG are transferred to IgG-free medium, they release in the form of low molecular weight breakdown products only some 30–60% of the stored fluorescein. There are several possible explanations for this observation. Some IgG molecules could be resistant to digestion, possibly as a result of a modification suffered in the course of fluorescein labeling; a fragment of some FC IgG molecules, or of all, could be indigestible; or labeling could have been heterogeneous, and the fluorescein molecules retained by the cells could be attached to a minor component of the IgG preparation rendered resistant to digestion by excess labeling. On the other hand, it is also possible that the IgG is itself digestible by lysosomal enzymes, and that part of it is stored either in inactive lysosomes, or in a nonlysosomal compartment—for instance, phagosomes that have failed to fuse with a lysosome, or components of the Golgi system, which morphological investigations have shown to be accessible to pinocytized macromolecules under certain conditions (7). These alternative explanations deserve special attention because the mechanism of IgG unloading on which our recycling model rests depends on an immunological type of binding between the plasma membrane ligand, which is a rabbit anti-rat plasma membrane IgG, and the stored fluorescein-labeled IgG, which is a goat anti-rabbit IgG IgG. Therefore, only F IgG molecules still capable of recognizing their antigen can be unloaded in this manner. Storage in a digestively inactive site would obviously favor retention of this property.

Experiments described in the present paper were designed to distinguish among these various possibilities. They lead to the conclusion that digestively active lysosomes are the sole storage site of the labeled material endocytized by the cells, and that the poor digestibility of the Fab portion of part of the IgG molecules taken up is largely responsible for the intracellular retention of some of the label.

MATERIALS AND METHODS

Materials

Experiments were carried out essentially as previously described (12, 13), with nonelicited (control) rabbit IgG (C IgG) first acetylated with [^{3}H]acetic anhydride (A) and then labeled with fluorescein isothiocyanate (F), as pinocytic substrate. Labeling was monitored to attach one to two molecules of acetate, and one to three molecules of fluorescein per IgG molecule. To remove any free label, FAC IgG was freshly filtered on Sephadex G-25 before use.

Kinetic Experiments

Except for minor changes, the experiments were conducted as before (12, 13), on fibroblasts incubated at 37°C in Eagle's medium containing 10% newborn calf serum and 100 μg/ml FAC IgG, or at 4°C in a similar medium buffered at pH 7.2 with 10 mM HEPES. At the end of incubation, the medium was removed, and the cells were washed four times with PBS (phosphate-buffered saline: 0.14 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4-KH_2PO_4, pH 7.4; 1 ml per 20-cm^2 dish surface), and dissolved in 3 ml of 1% Na deoxycholate adjusted to pH 11.3 with NaOH. Radioactivity, fluorescence, and protein were measured on the cell lysates. The collected media were assayed for degradation products, as separated either by precipitation of the proteins by 15% TCA followed by 30 min of centrifugation at 2,200 rpm, or by gel filtration through a 1.6 × 40 cm column of Sephadex G-100 with PBS as eluant. The TCA supernates were assayed for radioactivity and the Sephadex fractions for fluorescence. In the latter case, readings made on the low molecular weight fractions having an elution volume comparable to that of free fluorescein were pooled. As will be shown, fragments of intermediate size were never detected in the medium.

Gel Filtration Analysis of Cells and Media

After incubation and washing as in the kinetic experiments, the cells were dissolved in 2% Triton X-100 in PBS. The cell lysates were centrifuged 15 min at 3,000 rpm, and the supernates, containing >90% of the labeled material, were applied to a Sephadex G-150 column. Elution was carried out with PBS, and the collected fractions were assayed both for fluorescence and radioactivity. The media were clarified, fractionated, and analyzed in the same way.

In Vitro Digestion

FAC IgG (1 mg/ml) was incubated at 37°C in 0.1 M acetate buffer, pH 4.8, containing 10 mM cysteine, in the presence of a rat liver lysosomal extract prepared as described by Trouet (14, 15). At regular intervals the mixture was analyzed by gel filtration, as above.

Fab and Fc fragments of FAC IgG were prepared by papain digestion, and separated on carboxymethyl cellulose, as described by Porter (11).

Immunoprecipitation

After incubation with FAC IgG, cells were washed and dissolved in PBS/2% Triton X-100; 0.1 ml of cell lysate was incubated for 3 h at 25°C with 0.1 ml of goat anti-rabbit IgG, -rabbit Fab, or -rabbit Fc antiserum. Then 2.5 ml of rabbit anti-goat IgG antiserum was added, and the mixture incubated for another 16 h at 4°C. The mixture was centrifuged for 1 h at 3,000 rpm and the pellet assayed for radioactivity. More than 94% of the radioactivity present in a cell lysate to which FAC IgG was added in amounts comparable to the accumulation levels observed in the kinetic experiments is precipitated in this manner. Nonspecific precipitation was determined similarly, with the goat antiserum replaced by a nonimmune goat antiserum; this value was subtracted from all results. Goat anti-rabbit IgG antiserum was obtained from Miles Laboratories (Slough, England). Goat anti-rabbit Fab or -rabbit Fc antisera were kindly offered by Dr. J. P. Vaerman of this Institute; they were all monospecific as checked by immunoelectrophoresis.

Cell Fractionation Experiments

For cell fractionation experiments, the fibroblasts were washed four times with PBS (10 ml per 180-cm^2 flask), and then harvested and fractionated by isopycnic centrifugation as previously described (12, 13, 16), except that a VTI 50 rotor (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.) was used. Galactosyltransferase activity was assayed according to Beaufay et al. (1).

Chloroquine Uptake

Cells were exposed to medium containing 100 μM chloroquine, collected, washed, and lysed as in the kinetic experiments. Chloroquine was measured fluorometrically on the lysates, with excitation and emission wavelengths of 340 and 370 nm, respectively. It was verified that FAC IgG and chloroquine did not affect each other's fluorometric assay.

RESULTS

Distribution of Label

Results summarized in Fig. 1 show that, with rare exceptions, FAC IgG samples were equally labeled with fluorescein and with [^{3}H]acetate. This was true irrespective of the subcellular localization and degree of processing of the samples. The only significant heterogeneity was observed in some low molecular weight breakdown products of minor importance (Fig. 1 *C*).

In view of the data of Fig. 1, all results are expressed in terms of the averaged weight equivalent of original IgG, as derived from fluorescence and from radioactivity measurements. It is realized that these weights are strictly correct only for intact IgG molecules, because breakdown products could have different labeling densities. This is indeed so for Fc and Fab fragments. As shown in Table I, the labeling density of the Fc part of the FAC IgG molecule is almost twice that of the Fab part. This difference will be taken into account in the interpretation of the results.

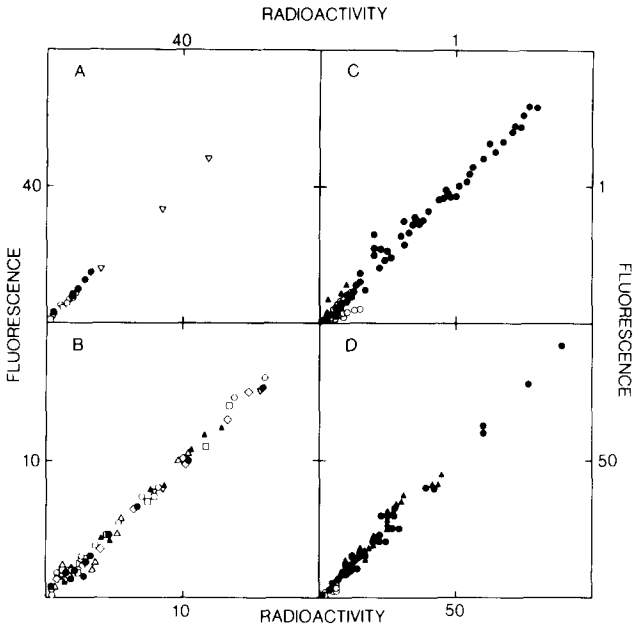

FIGURE 1 Parallel labeling of FAC IgG. Graphs show fluorescence of various samples vs. their radioactivity, both expressed in terms of the IgG amounts with which they were originally associated. Units in each panel are ordinate of corresponding figure. (*A*) Kinetic experiment of Fig. 2: □, 4°C binding; ●, 37°C accumulation; ▽, digestion; ○, washout. (*B*) Fractionation experiments: ●, Fig. 6 *B*; ○, Fig. 6 *C*; ▽, Fig. 6 *D*; □, Fig. 8 *A*; △, Fig. 8 *B*; ▲, Fig. 8 *C*; ◇, Fig. 8 *D*. (*C*) Gel filtration of material stored in vivo, Fig. 3: ●, 1st peak or peaks (80–130 ml); ○, 2nd peak (140–170 ml); ▲, 3rd peak (220–260 ml). (*D*) gel filtration after "in vitro" digestion, Fig. 5: symbols as in *C*.

TABLE I

Distribution of Label within FAC IgG Molecule

	Material Relative labeling density*	
Material	Radioactivity	Fluorescence
Whole IgG	1.0	1.0
Fc	1.43	1.46
Fab	0.79	0.77

* Label per milligram of protein, relative to IgG.

Kinetics of Uptake and Processing of FAC IgG

In Fig. 2 are shown the results of three experiments in which the cellular uptake and digestion of FAC IgG were followed over 3 d. These results confirm our previous surmise (12) that cellular accumulation of labeled material levels off not because uptake tends to stop, but because it is compensated by breakdown. In actual fact, the rate of clearance of FAC IgG from the medium remains essentially constant throughout the whole observation period. The reason why the rate of FAC IgG uptake slows down progressively (see Fig. 2, slope of *U* curve) is that, with the cells-to-medium ratio adopted in these experiments, the concentration of FAC IgG in the medium decreases progressively as a result of cellular uptake (see Discussion).

An important feature, not previously detected because of a shorter duration of exposure of the cells to FAC IgG, is that the cellular accumulation of labeled material does not reach a true plateau, but rather continues to rise, slowly but steadily, during the whole incubation period. This rise is all the more significant because, with normal processing, the steady-state level of intracellular labeled material would be expected to

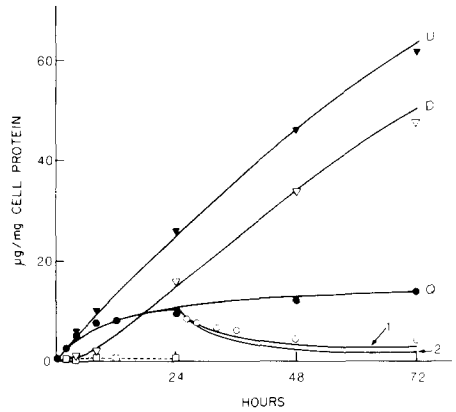

FIGURE 2 Kinetics of uptake and digestion of FAC IgG by fibroblasts. Averaged results of three experiments in which cells (3.24 mg protein) were incubated in 50-cm^2 petri dishes with 5 ml of medium containing 100 μg of FAC IgG/ml, either at 4° or 37°C. After various intervals, cells were washed, dissolved, and analyzed for fluorescence, radioactivity, and protein. The collected medium was assayed for TCA-soluble radioactivity and low molecular weight fluorescent degradation products. Results shown are the averages of the radioactivity and fluorescence measurements (expressed in terms of the weight of the corresponding IgG), which were always in close correspondence with each other (Fig. 1 *A*). □, Cell-associated material, 4°C. ●, *Q*: cell-associated material, 37°C. ▽, *D*: digestion products in medium, 37°C. ▼, *U*: total uptake, sum of *Q* + *D*. ○: Cell-associated material in cells transferred to FAC IgG-free medium after 24-h exposure to FAC IgG, 37°C (washout experiment). Curves shown represent kinetic equations (see Appendix), with C_0 = 100 μg/ml, P = 3.24 mg of protein/dish, V = 5 ml, F = 0.0113 ml/mg of protein/h, k = 0.115 h^{-1}, k' = 0.0073 h^{-1}, and α = 0.88. Decay curves (washout experiment) represent first-order breakdown of intracellular digestible material estimated by dualistic theory (*1*) or by monistic theory (*2*) (see Appendix).

decrease with time, in parallel with the decrease in the rate of FAC IgG uptake. This finding is important, because a major unexplained problem raised by our earlier results was how to reconcile what appeared to be the establishment of a steady state with the evidence indicating irreversible accumulation of indigestible material. Our current results are compatible with the occurrence of both processes. Indeed, it is clear from the shape of the observed curves that some sort of near-steady-state situation between uptake and digestion of IgG obtains after some hours. At the same time, the slow storage of indigestible material is suggested by the continuing rise of the accumulation curve, and is further confirmed by the results of the washout experiment, in which cells were transferred to FAC IgG-free medium after a 24-h exposure to FAC IgG. As illustrated in Fig. 2, only about half the accumulated label could be unloaded by digestion during the subsequent 48 h, confirming earlier results of a similar nature (12, 13).

Analysis of Intracellular FA-labeled Material

To investigate the nature of the material that remains trapped intracellularly, we have extracted cells after various periods of exposure to FAC IgG or of washout after a 24-h incubation with FAC IgG, and analyzed their contents in labeled material by Sephadex gel filtration. The results are depicted in Fig. 3. Comparing first the 24-h (*A*), 48-h (*B*) and 72-h (*C*) patterns, we see that the main change with time is the progressive development of shoulders on the low molecular weight side of the main peak, leading to the appearance of a new peak in the

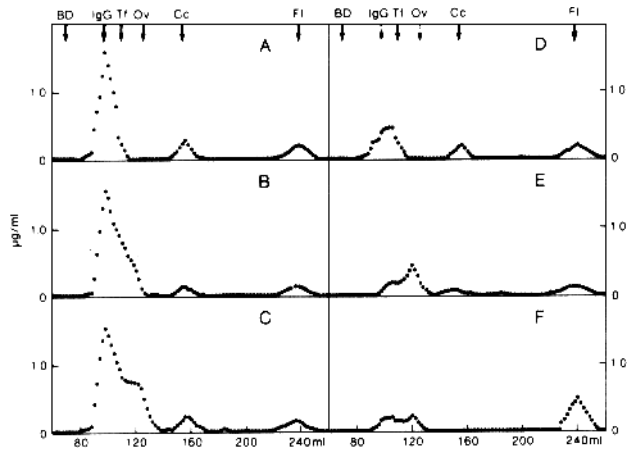

FIGURE 3 Gel filtration of cell-associated label. Cell lysates were applied on a 1.6 × 95 cm column of Sephadex G-150, and eluted in 2-ml fractions with PBS. Ordinate shows average of radioactivity and fluorescence of fractions, both expressed in terms of the corresponding amount of IgG. Arrows on top of the graphs indicate elution voume of substances used to calibrate the column: *BD*, blue dextran (70 ml, 2×10^6 mol wt); *IgG*, FAC IgG (98 ml, 1.6×10^5 mol wt); *Tf*, transferrin (110 ml, 7.7×10^4 mol wt); *Ov*, ovalbumin (126 ml, 4.5×10^4 mol wt); *Cc*, cytochrome *c* (154 ml, 1.3×10^4 mol wt); *Fl*, fluorescein (240 ml, 3.32×10^2 mol wt). Cells were incubated with FAC IgG for 24 (*A*), 48 (*B*), or 72 h (*C*), or incubated with FAC IgG for 24 h, washed, and reincubated in the absence of FAC IgG for 24 h (*D*), or 48 h (*E*), or were treated as for D and extracted, the extract being subjected to a 24-h incubation in vitro in the presence of a lysosomal extract, as described in the legend of Fig. 5 (*F*). Respective amounts of protein (mg) and labeled material (μg IgG) in each sample were: *A*, 2.7 and 26.5; *B*, 2.8 and 36.8; *C*, 2.8 and 39.9; *D* and *F*, 2.6 and 13.6; *E*, 2.7 and 13.2.

50,000 mol wt region, apparently by way of a 100,000 mol wt intermediate. The ascending arm of the first peak, which most closely reflects the elution of intact IgG molecules, is virtually unchanged. Obviously, the cells do not store increasing amounts of intact FAC IgG once they have reached a near-steady state between uptake and digestion. What they accumulate are partly digested breakdown products of about one-third the molecular weight of intact IgG. The other two peaks, in the 13,000 and 300 mol wt regions, do not change with time, and therefore presumably represent steady-state levels of digestive intermediates or products.

The above interpretation is further supported by the analysis of the cells subjected to a 24-h (*D*) or 48-h (*E*) washout period. The main change after a 24-h washout is a large reduction of the intact IgG peak. The cells are left with partly digested breakdown products, most of which occupy the region of apparent 100,000 mol wt. During the subsequent 24-h washout (*E*), there is a slow conversion of 100,000 to 50,000 mol wt material; but further breakdown of these fragments is arrested. There is no increase in the two lower molecular weight peaks; nor, as shown in Fig. 2, are degradation products discharged extracellularly. In contrast, 24-h material can still be further degraded by lysosomal enzymes in vitro, partly to material of ~50,000 mol wt, as in vivo, but also partly to very low molecular weight products that do not appear in vivo, either intracellularly or extracellularly (Fig. 3 *F*).

To try to identify the indigestible 50,000 mol wt material that accumulates in the cells, we have analyzed cell extracts immunologically. Results listed in Table II show that after a 24-h continuous exposure of the cells to FAC IgG, ~50% of the accumulated material reacts with antisera against intact IgG and against its two main parts. This corresponds most likely to essentially intact FAC IgG molecules, in agreement with Fig. 3 *A*. An additional 25% reacts with anti-IgG and anti-Fab, but not with anti-Fc. This material probably occupies the 100,000 mol wt shoulder in the elution pattern, and consists of F(ab')2-type fragments, which dissociate slowly into Fab'-type fragments (50,000 mol wt peak in Fig. 3 *B* and *C*). The remainder is so far degraded as to react with none of the three antisera, and presumably appears mostly in the 13,000 and 300 mol wt peaks.

After a subsequent 24-h washout period (Table II), "intact" IgG has fallen to ~20%, reactive Fab pieces remain at ~25%, and the unreactive degradation products have risen to >50%. These results are entirely consistent with the elution pattern of Fig. 3 *D*.

It appears, therefore, that the indigestible fragments that accumulate in fibroblasts exposed to FAC IgG originate largely from the Fab part of the molecule. Because this part is less densely labeled than the Fc part (Table I), our method of calculation, which is based on the average labeling density of whole IgG, leads us to underestimate the amount of material that remains stored intracellularly. This will be corrected for in the Discussion.

Analysis of the Culture Medium

Gel-filtration analysis of FAC IgG–containing culture media that have been in contact with cells for various periods reveals the presence of two clearly separated peaks (Fig. 4). The first

TABLE II

Immunoprecipitation of Cell-associated Material

Antiserum	% Immunoprecipitation	
	24-h continuous incubation*	24-h incubation + 24-h washout‡
Goat anti-rabbit IgG	74.3	51.7
Goat anti-rabbit Fc	52.5	21.3
Goat anti-rabbit Fab	78.3	58.2

* Cell lysate as in Fig. 3 *A*.
‡ Cell lysate as in Fig. 3 *D*.

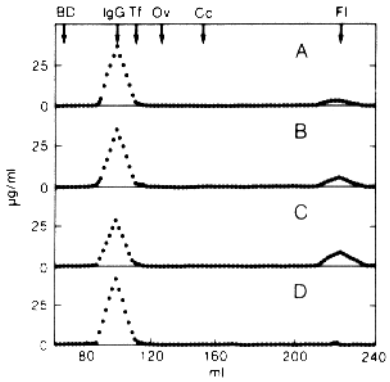

FIGURE 4 Gel filtration of culture media. 5 ml of culture medium containing 100 μg of FAC IgG/ml were incubated at 37°C for 24 (*A*), 48 (*B*), or 72 h (*C*) in the presence of fibroblasts, or for 72 h in the absence of cells (*D*). The cell contents in labeled material (expressed as *IgG*) were: 10.6 (*A*), 12.2 (*B*), and 13.6 (*C*) μg/mg of protein. Gel filtration analysis, was as in Fig. 3. 95% of the label applied on the column was recovered in the fractions.

one elutes as intact IgG and its area decreases progressively with time. The second peak elutes from the column like free fluorescein and its area increases reciprocally to the decrease of the first peak. No peak corresponding to FA-labeled material of intermediate molecular weight is observed, indicating that the cells are unable to reject partly degraded IgG, including its indigestible residues.

In Vitro Digestion of FAC IgG

In the hope of mimicking lysosomal digestion in vitro and of analyzing its mechanism in greater detail, we incubated FAC IgG preparations for up to 6 d in the presence of a lysosomal extract, under conditions similar to those adopted by Coffey and de Duve (2) for the study of lysosomal proteolysis, except that the pH was adjusted to 4.8, the pH value estimated by Ohkuma and Poole (10) to prevail in mouse macrophage lysosomes. The mixture was analyzed at daily intervals by Sephadex gel filtration and immunoprecipitation.

The results of these experiments, illustrated in Fig. 5, reveal important similarities between the in vitro and in vivo degradation of FAC IgG. We see that one-half of the label appears rapidly in very low molecule weight products, whereas the remainder appears more slowly in 50,000 mol wt material by way of 100,000 mol wt material. Immunological analysis (not shown) has confirmed that these high molecular weight materials originate from the Fab part of the molecule, as they do during in vivo digestion.

FAC IgG digestion is, however, less extensive in vitro than it is in vivo, yielding hardly >50% of the label as products of very low molecular weight, against >80% in vivo. Inactivation of the enzymes is not responsible for this, because addition of fresh lysosomal extract after 2 d did not alter the final pattern (Fig. 5 *F*). Oddly enough, material left in the cells after a 24-h washout period and apparently resistant to in vivo degradation into very low molecular weight products, proved partly susceptible to in vitro breakdown (Fig. 3 *F*).

Subcellular Localization of Stored FAC IgG and Breakdown Products

Results represented in Fig. 6 confirm our previous observations showing that the subcellular distribution pattern of stored label is practically identical to that of the lysosomal marker *N*-acetyl-β-glucosaminidase, both under pseudo-steady-state conditions and after a 24-h washout period. This pattern is almost entirely dissociated from that of the Golgi marker galactosyltransferase, which itself retains its typical low density profile in cells exposed to FAC IgG for 24 h, and in cells so exposed subjected to a subsequent 24-h washout period. Some labeled material occurs in the Golgi region of the gradient, but as part of a diffuse trail almost certainly attributable to losses from the lysosomes in the course of centrifugation. These results argue against a significant participation of the Golgi system in the storage of FAC IgG.

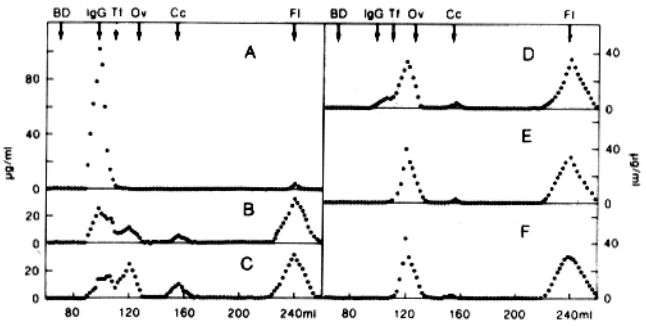

FIGURE 5 In vitro digestion of FAC IgG by lysosomal enzymes. Gel filtration analysis, as in Fig. 3, of reaction mixtures containing, in a total volume of 1.0 ml, 1 mg of FAC IgG, 100 μg of purified lysosomal enzymes, 0.1 M acetate buffer (pH 4.8) and 10 mM cysteine, incubated under sterile conditions at 37°C for 0 (*A*), 1 (*B*), 2 (*C*), 3 (*D*), or 6 (*E*) d, or for 6 d with addition of fresh enzyme on the 3rd d (*F*).

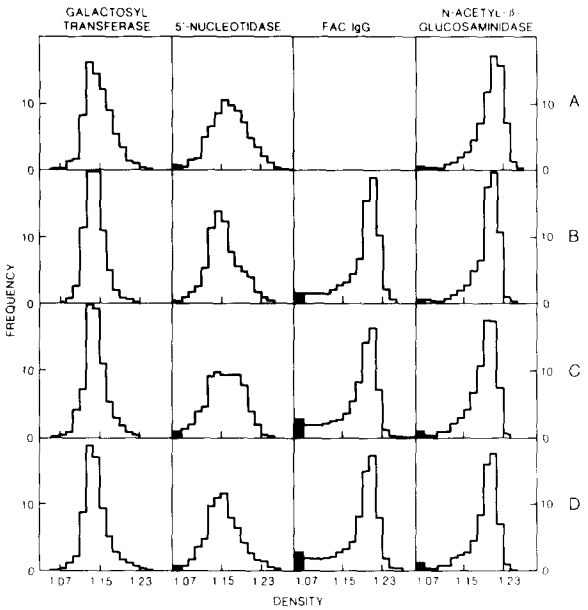

FIGURE 6 Isopycnic centrifugations of postnuclear supernate (PNS) from control fibroblasts (*A*), from cells incubated for 24 h at 37°C with 100 (*B*) or 250 (*C*) μg of FAC IgG/ml, or from cells incubated for 24 h at 37°C with 100 μg of FAC IgG/ml and then reincubated for 24 h at 37°C in the absence of FAC IgG (*D*). The cell contents in labeled material (expressed as *IgG*) were 9.3 (*B*), 22.8 (*C*), and 4.7 (*D*) μg/mg of cell protein. Recoveries ranged from 81 to 108%. Graphs represent normalized density frequency distribution patterns of assayed enzymes and materials.

Effect of Chloroquine

Wibo and Poole (17) have shown that cells can concentrate enormous amounts of chloroquine in their lysosomes, presumably as a result of proton trapping of this dibasic drug in the acidic lysosomal milieu (3). As a result of chloroquine accumulation, the lysosomes take up water and swell, and their equilibrium density in a sucrose gradient decreases (17). Findings illustrated in Fig. 7 show that cells exposed to FAC IgG for 24 h and subsequently subjected to a 24-h washout period concentrate chloroquine as strongly as do control cells. As shown in Fig. 8, cells so treated undergo the same decrease in lysosome density as do control cells upon exposure to chloroquine, and the labeled FAC IgG breakdown products stored in the cells accompany the lysosomal marker in its density shift.

DISCUSSION

Limitations of Experimental Approach

Our main purpose in carrying out the experiments described in this paper was to identify the nature and intracellular storage site of the fluorescein-labeled material that fibroblasts store

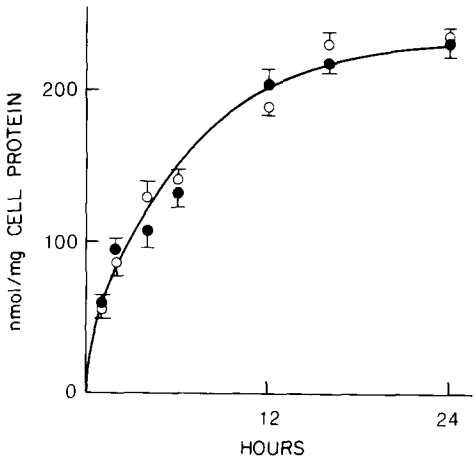

FIGURE 7 Kinetics of the uptake of chloroquine by rat fibroblasts. Cells (~400 μg of cell protein) were first incubated (●) or not (○) for 24 h at 37°C in 1 ml of medium containing 100 μg of FAC IgG/ml, then incubated for 24 h at 37°C in the absence of FAC IgG, and finally reincubated for different durations in 1 ml of medium supplemented with 100 μM chloroquine. At the end of each experiment cells were washed, dissolved in 3 ml of 1% deoxycholate, and assayed for chloroquine fluorescence and protein. Mean of three different experiments ± SD.

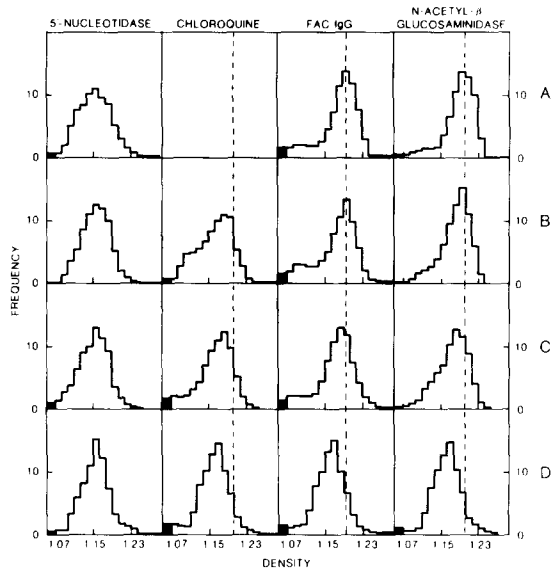

FIGURE 8 Isopycnic centrifugation of PNS from fibroblasts incubated for 24 h at 37°C with 100 μg of FAC IgG/ml, incubated for another 24 h at 37°C in the absence of FAC IgG, and then fractionated, as such (*A*) or after incubation for 1 (*B*), 16 (*C*), or 24 h (*D*) at 37°C in a medium supplemented with 100 μM chloroquine. The cells' content in labeled material (expressed as IgG) was 4.8 (*A*), 4.9 (*B*), 4.6 (*C*), and 4.8 (*D*) μg/mg of cell protein; their chloroquine content was 63 (*B*), 210 (*C*), and 216 (*D*) nmol/mg of cell protein. Recoveries ranged from 88 to 109%. Representation as in Fig. 6. Broken vertical lines indicate position of lysosomal peak in cells that have not been exposed to chloroquine.

upon exposure to FC IgG and especially retain intracellularly at the end of a washout period when release of stored label into the medium has come to a stop. Specifically, the question to be settled was: are we dealing with essentially intact and intrinsically digestible FC IgG molecules stored in a nonlysosomal site or in digestively inactive lysosomes or with indigestible FC IgG molecules or molecular remnants stored in authentic lysosomes?

Because our earlier experiments had depended only on fluorescence measurements for the detection of FC IgG and its degradation products, we have used doubly labeled FAC IgG to guard against errors resulting from some peculiarity of fluorescein labeling. Except for a minor breakdown product of approximately 13,000 mol wt, which contained two to three times more [^{3}H]acetate than fluorescein, the two labels remained closely correlated in all analyzed samples (Fig. 1). Considering that the labels were attached to the IgG sequentially as well as sparsely, this finding allows us to exclude any gross labeling artifact.

The use of labeled materials for the study of protein digestion does, however, have its limitations. As shown by Table I, the Fc part of the IgG molecule was almost twice as densely labeled, both with acetate and with fluorescein, as was the Fab part, a reflection no doubt of a greater availability of reactive lysine residues in the Fc portion. Further breakdown of the molecule could well generate greater inequalities in labeling density. It may even, as we have seen result in a partial separation of the two labels (see the minor 13,000 mol wt component in Fig. 1 *C*).

Fortunately, very few labeled breakdown products of intermediate size were found to accumulate either in vivo or in vitro. The main products were F(ab')2- and Fab'-type fragments, for which a suitable correction can be made on the basis of the data of Table I (see below), and compounds of very small molecular weight eluting with free fluorescein, and consisting almost certainly of acetyl-lysine and fluorescein-lysine. These are very unlikely to be carved out selectively, and may safely be taken as indicators of complete breakdown of the part of the IgG molecule from which they originate. This assumption will be made in the following discussion.

Digestion of FAC IgG in Vivo and in Vitro

To take into account the peculiarities of our system, we have modified our previous kinetic equations in the manner explained in the Appendix. As illustrated by the curves of Fig. 2, we obtain a satisfactory fit between theory and data for FAC IgG uptake and digestion, by assuming a clearance rate, F, of 11.3 μl/mg of cell protein per h, a first-order constant, k, for the digestive process of 0.115 h^{-1} (two values consistent with earlier results), and a coefficient of digestibility, α, of 0.88. The latter value needs, however, to be corrected for nonuniform labeling of the IgG (Table I). According to the results of Fig. 3 and of Table II, we may take it that the nondigested fraction originates mainly from the Fab part of the molecule. Therefore, what is not digested represents not 12%, but 12/0.78 = 15.4% of the IgG molecules, or ~25% of their Fab part. Should all FAC IgG molecules be degraded to the same incomplete extent, we should be left with fragments of 25,000 mol wt or less. In fact, however, the fragments that accumulate intracellularly have twice this size; they behave like Fab'-type fragments, which themselves originate from dimeric F(ab')2-type fragments (Fig. 3). Therefore the FAC IgG molecules are broken down unequally: ~75% are completely digested, and the rest have their Fc part digested, but not their Fab part. There can be no doubt that 75% of the Fab parts are indeed digested, because the alternative possibility of their being discharged into the medium can be excluded (Fig. 4).

The results of the washout experiments are somewhat at variance with the above conclusions. Whatever assumption is made about the nature of the accumulating material, the cells break down significantly less than predicted (Fig. 2). The

discrepancy is even greater if correction is made for the low labeling density of the stored material. We suspect that some peculiarity of the washout experiment must be responsible for the inability of the cells to drain their digestible pool as effectively as they do under steady-state conditions. As far as can be made out, the block concerns a small amount of essentially intact FAC IgG molecules remaining after a 24-h washout (Fig. 3 *D* and Table II), of which a part, presumably the Fc portion, can still be completely degraded in vitro (Fig. 3 *F*), but no longer in vivo (Fig. 3 *E*). Note, however, that these molecules do not remain intact in vivo. After a further 24-h incubation in the absence of FAC IgG, they have been completely converted to fragments of either 100,000 or 50,000 mol wt (Fig. 3 *E*). Therefore, even this small FAC IgG pool, which represents less than one-tenth of the total taken up in 24 h, is exposed to proteolytic enzymes.

The pattern of in vitro digestion of FAC IgG by a lysosomal extract resembled the in vivo pattern, except that breakdown was less extensive. As illustrated in Fig. 5, about half the label remained in fragments of ~50,000 mol wt that could not be digested further, even with fresh enzyme, and that were identified immunologically as originating from the Fab part of the molecule. Correcting for labeling density (Table I), we find that the indigestible fraction amounts in weight to some 60–65% of the FAC IgG added, which is the proportion the Fab part occupies in the IgG molecule. In vitro, therefore, essentially all FAC IgG molecules yield Fab' fragments that are resistant to digestion, whereas only one-fourth do so in vivo.

Other workers before us have noted the resistance of the Fab part of the IgG molecule to digestion both in vivo (6) and in vitro (4, 5, 8, 9). To our knowledge, ours is the first report indicating that the Fab portion can be digested in vivo. It is somewhat surprising that only about three-quarters of the molecules have a digestible Fab part, but it is not inconsistent with known facts, because the Fab part contains the variable regions of the IgG molecule. It is possible that some variable sequences can be attacked by cellular proteases, and that others cannot. An alternative possibility is that some of the fibroblasts lack the necessary enzymes for digesting Fab fragments, as do the hepatic lysosome extracts used in our in vitro work, and perhaps also the guinea pig peritoneal polymorphonuclear leukocytes used by Ghetie and Sulica (6) in their studies of in vivo degradation of doubly labeled (^{131}I and fluorescein) rabbit IgG.

Subcellular Site of FAC IgG Storage and Digestion

The following facts point to the lysosomes as the principal and probably the sole site of FAC IgG storage and processing in fibroblasts (except for the initial passage through phagosomes): (*a*) In confirmation of earlier results (12, 13), stored label was found upon isopycnic density gradient fractionation to accompany closely the lysosomal marker *N*-acetyl-β-glucosaminidase under all conditions tested (Figs. 6 and 8). (*b*) The distribution pattern of the labeled material was almost entirely dissociated from that of the Golgi marker galactosyltransferase, and the shape of the latter distribution pattern was entirely unaffected by the uptake and processing of FAC IgG (Fig. 6), arguing against a possible involvement of the Golgi complex in the storage of FAC IgG. (*c*) Cells exposed to FAC IgG accumulated chloroquine as strongly as did control cells (Fig. 7), and this phenomenon resulted in identical shifts towards lower densities of the distribution patterns of FA-labeled material and of the lysosomal marker, accompanied by the stored chloroquine (Fig. 8), indicating that the storage site of labeled material shares with lysosomes the ability to concentrate chloroquine, and therefore the capacity to maintain the low pH believed to be needed for this process (3, 10, 17). (*d*) All FAC IgG molecules taken up by the cells suffer at least partial proteolytic degradation, and many are digested completely, as discussed above.

We conclude that the "storage compartment" shown in our earlier experiments to serve as intracellular transit station for recycling or shuttling plasma membrane fragments (13) consists of authentic, digestively active, lysosomes.

Implications for Lysosome Function

Our evidence for membrane recycing or shuttling has been described before (13). The present work has confirmed that, as suspected, the pieces of plasma membrane that return to the cell surface after pinocytic interiorization pass through the lysosome compartment. They do so in such a way as to be able of picking up ligands from the lysosome contents and delivering them to the extracellular medium.

The mechanisms involved in this phenomenon raise intriguing questions. We will consider only its general implications with respect to the functioning of lysosomes. It is obvious that, contrary to what has long been believed, lysosomes are not a metabolic dead end from which trapped molecules can escape only in the form of small diffusible digestion products, or, exceptionally, in certain cell types or under certain well-defined physiological or pathological circumstances, through bulk exocytic discharge. They are connected with the extracellular medium by means of mobile membrane patches acting as some sort of endless moving belt, and are capable of transporting materials in both directions.

When moving inwards, through endocytosis, the belt brings in from the outside various substances, including hormones, that have become bound to some surface receptor. Such substances, however, will be delivered to the lysosomal compartment; there they undergo digestion, but only to the extent that they become detached from their receptor—for instance, by exposure to the lysosomal acidity, or through enzyme action, or by some other agency prevailing in the intralysosomal milieu. If this detachment fails to occur, the membrane-bound substance will return to the cell surface, either intact or possibly altered in some ways by its exposure to the detersive lysosomal contents. The anti–plasma membrane antibodies used in our earlier experiments provide, in our opinion, a particularly striking example of strongly bound ligands that cycle almost endlessly in this manner. Membrane constituents may be expected to behave similarly. But the possibility that some of them become either detached or altered by lysosomal action cannot be excluded, and will have to be given consideration in future schemes.

In addition to membrane-bound ligands, endocytosis also brings to the lysosomes droplets of extracellular fluid—quite a sizable traffic, in some cases—and especially certain molecules, such as the fluorescein-labeled IgG used in our work, that are taken up with a remarkable selectivity without the participation of specific receptors. We have commented before on the puzzling aspects of this uptake process, the nature of which eludes us at the present time.

Particularly intriguing are the phenomena that may be associated with the return of the membrane patches from the

lysosomes to the cell surface. Most likely, they pinch off from the lysosomal surface in the form of closed vesicles. Therefore, all considerations of the inward traffic may well be applicable, *mutatis mutandis*, to the outward traffic. Substances—including modified exogenous constituents—may be transported from the lysosomes to the extracellular milieu as membrane-bound ligands, or as the contents of fluid droplets, or perhaps through some special mechanism. These possibilities clearly have many implications.

So far, little is known of the cellular itinerary followed by plasma membrane patches in their return journey. One would particularly like to know whether another transit station exists (for instance in the Golgi system, as certain experimental results suggest) where further processing, or repair, of transported ligands or membrane constituents may take place.

APPENDIX

Kinetic Analysis

Two new features must be introduced into the equations worked out previously (12): (*a*) the IgG concentration in the medium is not constant, but decreases progressively during incubation; and (*b*) digestion is incomplete.

Taking into account the first point, we obtain for the total amount of FAC IgG taken up at time t:

$$U_t = \frac{VC_0}{P}(1 - e^{-k't}), \tag{1}$$

in which: U_t is the amount of FAC IgG taken up, in micrograms per milligram of cell protein; V is the volume of medium, in milliliters; C_0 is the initial FAC IgG concentration, in micrograms per milliliter; P is the amount of cell protein per dish, in milligrams; $k' = (FP/V)h^{-1}$; F is the clearance rate of FAC IgG in milliliters per milligram of protein per hour; and t is the time, in hours.

Net accumulation Q_t', assuming complete digestion (equation *4* in reference 12), now becomes:

$$Q_t' = \frac{FC_0}{k - k'}(e^{-k't} - e^{-kt}), \tag{2}$$

in which Q_t' is expressed in micrograms per milligram of cell protein and k is the first-order constant of the digestive process, in hours^{-1}.

The amount of FAC IgG digested at time t, D_t', expressed in microgram per milligram of cell protein, is then given by:

$$D_t' = U_t - Q_t'. \tag{3}$$

To take into account the second point, we introduce a coefficient of digestibility, α, representing the proportion of the IgG taken up ending as label released into the medium. Then, the amount actually digested, D_t, is given by:

$$D_t = \alpha D_t' = \alpha\,(U_t - Q_t'), \tag{4}$$

and the total amount, Q_t, of label accumulated by the cells becomes:

$$Q_t = Q_t' + (1 - \alpha)(U_t - Q_t'). \tag{5}$$

The proportion of digestible and indigestible material making up Q_t depends on the mechanism responsible for lack of digestion. If we assume that a fraction α of the IgG molecules taken up are broken down completely, while the remainder is stored essentially intact, either because it is intrinsically indigestible or because it is conveyed to a cellular compartment lacking digestive ability (dualistic theory), then we have:

$$\text{indigestible material stored} = Q_i = (1 - \alpha)U_t; \tag{6}$$

$$\text{digestible material stored} = Q_d = \alpha Q_t'. \tag{7}$$

On the other hand, if all the IgG taken up is assumed to undergo digestion in the lysosomes, but only a proportion α of the products of digestion have properties such that they can be cleared out of the lysosomes and released into the medium (monistic theory), then:

$$Q_i = (1 - \alpha)(U_t - Q_t') \tag{8}$$

and

$$Q_d = Q_t'. \tag{9}$$

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