PATHWAYS FOLLOWED BY MEMBRANE RECOVERED FROM
THE SURFACE OF PLASMA CELLS AND MYELOMA CELLS*

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In general, the production of immunoglobulins by plasma cells (1–5) involves the
same operations and pathway as the production of secretory proteins by cells of
exocrine and endocrine glands (6, 7)—i.e., synthesis on attached polysomes followed by
segregation in the cisternal space of the rough endoplasmic reticulum (ER),† packaging
in the Golgi, and discharge by exocytosis. The main difference is that, whereas in
glandular cells secretory products are concentrated (up to 50–150 times [6–8]) and
packaged into morphologically recognizable secretion granules that can be stored
before discharge, there is usually little (if any) concentration or intracellular storage
of immunoglobulins (5). They appear to be packaged in dilute solution in small,
membrane-limited vesicles that continually release their content by exocytosis.
Because such vesicles cannot be distinguished morphologically from other vesicles (e.g.,
endocytic vesicles) with a different content, their identification as immunoglobulin
 carriers has been inferred from results obtained by cell fractionation (1), autoradiog-
raphy (9), and especially by immunostaining (10–12). In both immunoglobulin-
secreting cells and cells of exocrine and endocrine glands, secretion, therefore, involves
the continual insertion of a considerable amount of Golgi-derived membrane into the
plasmalemma at the time of exocytosis. In glandular cells, it has been repeatedly
demonstrated (13–15) that, after exocytosis, membrane is continually removed from
the cell surface by endocytosis to maintain a constant cell size. Evidence obtained
recently on several exocrine and endocrine cell types indicates that at least some of
the membrane retrieved from the cell surfaces fuses with multiple cisternae of the
Golgi stacks (14–18) and suggests that the recovered membrane may be reused in the
packaging of newly synthesized secretory products (15).

The purpose of this study is to determine whether or not membrane is similarly
retrieved from the surface of immunoglobulin-secreting cells and returned to the
Golgi complex. Because these cells do not concentrate their product and exocytosis is
a continual process, it seemed likely that membrane traffic (from Golgi to cell surface
and back) might be relatively extensive. If this assumption were proved to be correct,
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Immunoglobulin-secreting cells would represent a convenient system for investigating, in further detail, mechanisms and pathways of membrane recycling in secretory cells. Established cell lines are available, they provide the usual advantages of homogeneous cell populations in which all cells are uniformly accessible to experimental variables, they can be easily grown in culture, and they can be produced in quantities sufficient for cell fractionation and subsequent biochemical studies. With this in mind, we have used immunocytochemical procedures to localize the secretory compartment and various tracers to follow the pathway of membrane recovered from the cell surface, and have carried out studies on both mature plasma cells harvested from lymph nodes and myeloma cell lines. The results reveal an active and continuous membrane traffic from the cell surface to the Golgi complex and lysosomes in immunoglobulin-secreting cells, which is, in general, comparable to that previously documented in exocrine and endocrine cells (14–18).

Materials and Methods

**Media and Chemicals.** Cell culture media, sera, antibiotics, and Freund’s complete and incomplete adjuvant were obtained from Grand Island Biological Co., Grand Island, N. Y.; native and cationized ferritin (GF), from Miles Laboratories Inc., Elkhart, Ind.; horseradish peroxidase (HRP) (types II and VI), diaminobenzidine (DAB), and p-nitroacetohol sulfate were obtained from Sigma Chemical Co., St. Louis, Mo.; Fab fragments of sheep antibodies to mouse immunoglobulins coupled to peroxidase from the Pasteur Institute, Paris (19); t-[4,5-3H]leucine from Schwarz/Mann Div., Amersham, England (40–60 Ci/mM sp act). All other chemicals were of reagent grade. Ferritin with isoelectric points of narrow range were provided by Dr. Y. S. Kanwar (20). Native ferritin was dialyzed (in two to three changes of phosphate buffer) before use. The isoelectric points (pI) of HRP was determined by isoelectric focusing in Sephadex gel (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) (21). Both Sigma type II and type VI HRP exhibited several bands with the major band having an apparent pI of ~7.8 (22).

**Plasma Cells.** Normal plasma cells were obtained from rats immunized with HRP following the procedure of Miller et al. (23). Young male rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were immunized with 0.5 mg HRP (Sigma type VI) in either complete (first injection) or incomplete Freund’s adjuvant in each hind foot pad. The animals were primed several times and boosted 1 wk before the experiment. Both popliteal lymph nodes were excised under phenobarbital anesthesia (7.5 mg/100 g body wt), dissected free of fat, and transferred to Dulbecco’s phosphate-buffered saline (PBS) where they were cut into small pieces with a razor blade, and subsequently teased with a pair of fine forceps. The teased tissue was then filtered successively through a 250-μm, followed by 70-μm, nylon mesh, which resulted in complete dispersion into a cell suspension. The cells were washed three times in PBS and resuspended in Dulbecco’s modified Eagle’s medium (DMEM). Siliconized tubes and pipettes were used to prevent sticking. Immunodiffusion carried out on serum from immunized animals showed high titers of precipitating antibodies against HRP.

**Myeloma Cell Lines.** Three different mouse myeloma cell lines were studied: RPC 5.4 and X63 Ag 8 were obtained as a gift from Dr. F. Ruddle, and MOPC 315 as a gift from Dr. M. Armstrong (both of Yale University, New Haven, Conn.). Cells were grown in tissue culture bottles or Petri dishes in DMEM without sodium pyruvate, which contained 4,500 mg glucose/liter and was supplemented with nonessential amino acids, penicillin (1,000 U/100 ml), streptomycin (10 mg/100 ml), and either 20% heat-inactivated horse serum (X63 Ag 8) or 13% heat-inactivated fetal bovine serum (RPC 5.4 and MOPC 315). Cells were harvested during exponential growth.

**Labeling of Cells with Tracer Proteins.** Cells were washed twice (by sedimentation and resuspension) in DMEM before addition of tracers. Incubation of lymph node cells was carried out in siliconized conical glass centrifuge tubes at a concentration of 10–20 × 10^6 cells/ml. Incubation of myeloma cells was similarly carried out either in plastic or siliconized-glass...
centrifuge tubes or in small Petri dishes (which contained 2 ml medium) at a concentration of 5-6 × 10^6 cells/ml. Incubations were at 0°, 25°, or 37°C in an atmosphere of 93% air/7% CO₂ for periods of 2 min to several hours. CF was used at a concentration of 0.1 mg/ml in the plasma cell experiments and 0.05 mg/ml in the myeloma cell experiments. Some myeloma cells were also exposed to native (anionic) ferritin at a concentration of 5-10 mg/ml or to HRP (Sigma type II or type VI) at a concentration of 1.0 mg/ml. Still others were exposed to both CF and HRP as follows: they were incubated first with CF (0.05 mg/ml) at 0°C, after which they were sedimented (by spinning at 1,000 g for 3 min) at 0°C, and resuspended and incubated in HRP (1.0 mg/ml) at 37°C.

**Processing of Cells for Electron Microscopy.** Cells exposed to ferritin were fixed in suspension by adding one part 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), or Karnovsky's fixative (1.6% paraformaldehyde and 3% glutaraldehyde in 0.1 M cacodylate buffer) to one part medium. After fixation (1-2 h at room temperature), they were sedimented in a Beckman Microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), and the resultant pellets were postfixed in OsO₄ in acetate-veronal buffer (45 min at 4°C), dehydrated in ethanol, and embedded in Epon. Cells exposed to HRP were fixed in 1% glutaraldehyde for 1-2 h at 0°C, washed overnight in 0.1 M cacodylate buffer, and incubated in DAB (24), postfixed in OsO₄, and embedded for electron microscopy as already described.

**Aryl Sulfatase Tests.** Myeloma cells (RPC 5.4) were fixed (10 min at 4°C) by adding one part 1% glutaraldehyde in 0.1 M cacodylate buffer to one part medium, after which the cells were pelleted, and washed (by sedimentation and resuspension) in 0.1 M cacodylate buffer that contained 7% sucrose. Incubation was carried out for aryl sulfatase (90 min at 25°C) with p-nitrocatechol sulfate as substrate at pH 5.5, according to Bentfield-Barker and Bainton's (25) modification of Goldfischer's (26) method.

**Immunocytochemistry.** Lymph node or myeloma cells were fixed by adding one part Karnovsky's fixative to one part medium (20 min at room temperature), after which they were washed several times in Tris-buffered saline (to quench aldehyde groups), and pelleted in 3% warm (~40°C) agar. The agar was allowed to solidify, and the resultant agar-embedded pellet was then frozen in isopentane cooled down to its melting point (~160°C) in liquid nitrogen. Cryostat sections (1-8 μm) were prepared with a conventional cryostat at ~18°C and collected in PBS that contained 0.1% bovine serum albumin. Sections of lymph node cells were incubated in PBS that contained 10-20 μg/ml HRP (Sigma type VI) at room temperature for 1 h to demonstrate intracellular sites of anti-HRP antibodies (12), after which they were washed three times in PBS and incubated for 30 min in DAB-containing medium (24). Sections of myeloma cells were incubated for 1 h at room temperature in peroxidase-conjugated anti-mouse Fab, washed in PBS, and incubated in DAB, as described for sections of plasma cells to demonstrate intracellular sites of mouse immunoglobulins (27).

**Evaluation of the Effect of CF on Cell Viability and Secretion.** To determine whether or not CF had a toxic effect on myeloma cells, we carried out trypan blue exclusion tests, assayed lactic dehydrogenase activity (LDH) (28) released into the medium, and measured rates of secretion of labeled products (after pulse-labeling of the cells [5]) in myeloma cells incubated in the presence and absence of CF. The number of trypan blue-stained cells, the LDH detected, and the rate of release of labeled proteins into the medium varied from cell line to cell line and from one experiment to another, but in a given experiment there was little or no difference between results obtained on cells incubated with CF as compared with controls. In one typical experiment the amount of LDH found in the medium of cells incubated for 1 h with CF was 70 U/liter (± 26 SD) for RPC 5.4 cells and 14 U/liter (± 6.5 SD) for X63 Ag 8 cells, whereas the corresponding figures in controls were 77 (± 12.5 SD) and 12 U/liter (± 5.5 SD), respectively. The rate of cell death as estimated by LDH release amounted to ~0.1-0.5%/h. The rate of secretion from cells incubated with CF was 5.8%/hour (± 1.9) for cells of the RPC 5.4 cell line and 6.9%/hour (± 1.0) for X63 Ag 8 cells, and the corresponding figures from controls were 4.2 (± 1.1) and 5.1% (± 1.6), respectively.

**Results**

**Plasma Cells**

Although myeloma cells were used for most of our tracer studies, we also did some experiments with CF on plasma cells obtained from murine lymph nodes to determine
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(a) whether normal plasma cells behaved in the same way with respect to their handling of CF as cells of the anterior pituitary (15, 16) and thyroid (17) glands, and (b) whether or not myeloma cells maintained in culture behaved in the same manner as normal, nonmalignant plasma cells. As a source of plasma cells we used lymph node cells harvested from rats immunized with HRP because the secretory compartments of these cells (which contain anti-HRP immunoglobulins) can be easily labeled by incubating the cells with HRP (11, 12).

EXPERIMENTS WITH CF. When lymph node cells were incubated with CF at either 0° or 37°C, the tracer bound to the surfaces of both lymphocytes and plasma cells. Lymphocytes tended to clump together and occasionally showed capping, but aggregation or capping of mature plasma cells was never observed. CF bound to the cell membranes of plasma cells in a relatively uniform layer (Fig. 1), and when these cells were incubated at 37°C after binding, there was extensive uptake of the bound CF by endocytosis (Fig. 1). During incubation periods of 10-60 min there was a progressive increase in the amount of CF found in the Golgi region where it was located in small vesicles, in larger vacuoles, and in multiple stacked Golgi cisternae (Figs. 2-4). Once within these compartments the CF typically occurred as small aggregates that remained at least partly membrane associated. Small vesicles that contained CF were seen in the Golgi region as early as 5-10 min after addition of the tracer, and CF was observed in the stacked Golgi cisternae by 20 min. The uptake and fate of the tracer was the same as that demonstrated previously in anterior pituitary cells: namely, it binds to the cell membrane, is then internalized by endocytosis, and the resultant endocytic vesicles that contain the tracer eventually fuse with multiple stacked Golgi cisternae and presumptive secretory vacuoles in the Golgi region. In plasma cells, however, there are typically fewer recognizable lysosomes than in dissociated pituitary cells and relatively little evidence of uptake of CF into lysosomes.

LOCALIZATION OF INTRACELLULAR IMMUNOGLOBULINS (ANTI-HRP ANTIBODIES). When lymph node cells from rats immunized with HRP were sectioned in a cryostat and incubated first with HRP and then with DAB to reveal the intracellular localization of antibodies to HRP, ~5% of the cells, which were mature plasma cells, were stained. As reported by others (11, 12), there was a general and intense staining of the rough ER, including the perinuclear cisterna, as well as the Golgi cisternae and associated vesicles (Fig. 5). It is noteworthy that many of the small vesicles located on both the cis and trans side of the Golgi were also stained, but a few were not. The cell surfaces of most plasma cells were not stained. When cells were exposed to CF before staining, the tracer was often detected in Golgi cisternae that also contained stained immunoglobulins (Fig. 6), which indicates that the incoming endocytic vesicles carrying CF fuse with elements along the secretory pathway.

Myeloma Cells

MORPHOLOGY. A number of mouse myeloma cell lines were screened initially. Of these, three were chosen for use in these experiments because the cells have extensive, well-developed Golgi complexes; those selected were two IgG-secreting lines, RPC 5.4 and X63 Ag 8, and the IgA-secreting myeloma, MOPC 315. All three have a similar morphology and are characterized by a rather extensive rough ER that is dilated in many regions and contains virus particles (of the C-type) budding into the cisternal lumina. The rough ER is not as closely packed as in normal plasma cells (compare
Figs. 1-4. Portions of plasma cells fixed after incubation in CF (0.05 mg/ml). Fig. 1 shows that CF binds in a relatively uniform layer to the cell membrane (arrow) and is taken up in endocytic vesicles (ve). Fig. 2 shows that after incubation in CF for 60 min considerable CF is present in the Golgi region where it is located within vesicles (ve) or vacuoles (v) of varying size and within several Golgi (G) cisternae (arrows). Figs. 3 and 4 are higher magnification views showing CF molecules within several of the stacked Golgi cisternae (arrows) as well as in vesicles (ve) located nearby. er, rough ER; m, mitochondria; N, nucleus. Figs. 1, 3, and 4: × 75,000. Fig. 2: × 16,000.
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FIG. 5. Plasma cell from the lymph node of a rat immunized with HRP showing the distribution of anti-HRP immunoglobulin as demonstrated by immunostaining. Immunoglobulin is present throughout the rough ER (er) including the perinuclear cisternae (pc), in the stacked Golgi cisternae (G), and in small vesicles or vacuoles (v) in the Golgi region. Other small vesicles (ve) are not stained. The specimen was fixed in glutaraldehyde, sectioned on a cryostat, and incubated with HRP, followed by staining in DAB. m, mitochondria; N, nucleus. X 22,000.

FIG. 6. Golgi region from a plasma cell from the same experiment as in Fig. 5 except that the cell had been incubated with CF for 60 min before fixation and immunostaining. Immunoglobulin is present throughout the stacked Golgi cisternae (G). Note the presence of CF molecules in one cisterna that also contains immunoglobulins (arrow). X 75,000.
Figs. 2 and 7), and free polysomes are much more abundant. The Golgi complexes typically consist of three to six stacked cisternae found near one pole of the nucleus (Fig. 7). The X63 Ag 8 cells have the fewest (2–4) cisternae, and the MOPC 315 and RPC 5.4 have more (4–6). A few lysosomes are typically seen in the Golgi region, and

Fig. 7. Portion of a mouse myeloma cell (RPC 5.4) demonstrating the presence of an extensive Golgi complex (G) consisting of three to six stacked cisternae. A centriole (ce), a lysosome (ly), and numerous small vesicles (ve) are seen on the trans (concave) side of the Golgi. A number of dilated cisternae of the rough ER (er) are present. Typically, some of them contain viral particles as seen here to the right (c). Transitional vesicles (tr) are present on the cis side of the Golgi. The mitochondria (m) are typically large with a matrix of low density and relatively few cristae. N, nucleus. × 15,000.
many transitional vesicles are present on the cis side of the Golgi where they often occur in clusters closely associated with the rough ER (Fig. 9).

**Experiments with CF.** Initial experiments showed that (a) when the cells were incubated with CF in the presence of serum, the tracer clumped and formed large aggregates, and (b) capping often occurred when the cells were incubated at 37°C in the presence of a relatively large concentration of CF (>0.2 mg/ml), whereas no capping was observed when the concentration was 0.05 mg/ml or less. Accordingly, subsequent experiments were carried out with serum omitted at a tracer concentration of 0.05 mg/ml.

When CF was added to a suspension of cells, it immediately bound to the cell surfaces, and the cells had a tendency to agglutinate into clumps. CF bound strongly to the cell membrane regardless of the temperature at which binding was done; however, its distribution varied with the binding temperature. It was relatively uniformly distributed along the cell surfaces when binding was carried out at 0°C, but its distribution was more uneven and patchy when binding was carried out at room temperature or at 37°C (Fig. 8). When cells were subsequently incubated at 37°C in the presence of CF, the tracer was taken up by endocytosis. As in the case of normal plasma cells, at 2-10 min it was located mainly at the cell periphery either in small vesicles (some of which were coated) or in invaginations of the cell membrane (Fig. 8), and, after 10 min, it was seen in increasing quantities in the Golgi region where it was initially located in small vesicles or in larger lysosomal structures (vacuoles or multivesicular bodies) (Fig. 9). Later (after 20 min), it was also seen in the stacked Golgi cisternae in cells of the X63 Ag 8 and RPC 5.4 cell lines, but not in MOPC 315 cells. The amount of CF found in the Golgi cisternae of the former two lines increased up to 40-60 min of incubation, at which time it was typically located in multiple stacked cisternae (Fig. 10). At this time it was also seen in small vesicles and in lysosomes as well as in tubular structures located in close proximity to lysosomes (Fig. 10) in cells of all three myeloma lines. In favorable sections some of these tubular structures were observed to be in continuity with lysosomes (Fig. 11). As in the case of the plasma cells after internalization, the CF had a tendency to aggregate into clumps or clusters that typically remained partly associated with the membrane of the receiving compartments.

The fact that there was variability in the findings among the major different cell lines with regard to the presence of CF within Golgi cisternae was interesting. We can provide no explanation for this finding; however, because CF was regularly found in multiple cisternae of the Golgi stacks in nonmalignant cells from lymph nodes, as well as in X63 Ag 8 and RPC 5.4 myeloma cells, it appears that the behavior of MOPC 315 myeloma cells is not typical, and, therefore, we did not use them for subsequent tracer experiments.

**Experiments with HRP.** Cells incubated in HRP showed extensive uptake of the tracer in small endocytic vesicles where it was found staining the content of such vesicles. This is in keeping with the fact that HRP is usually considered a reliable content marker for fluid-phase pinocytosis (29). After a 1-h period of incubation, the intracellular distribution of the tracer was different from that of CF: HRP was observed only in lysosomes (large vacuoles and multivesicular bodies) and in smaller vesicles and tubular structures in the vicinity of lysosomes. It was never seen in the stacked Golgi cisternae of these cells (Fig. 12).
Figs. 8 and 9. Mouse myeloma cells (RPC 5.4) fixed after incubation in CF for 10 min at 37°C. Fig. 8 shows CF molecules distributed in patches along the cell membrane (cm) and concentrated in a coated invagination of the surface membrane (arrow) where it adheres to the membrane. m, mitochondria; er, rough ER. Fig. 9 shows the distribution of CF in the Golgi region. At this time CF is present in numerous small vesicles (ve), in a multivesicular body (mv), and in lysosomes (ly), all of which are located on the trans side of the Golgi stack. No ferritin is seen in the Golgi cisternae (G) at this early time. Numerous transitional vesicles (tr) are present on the cis side of the Golgi between the rough ER (er) and the Golgi cisternae (G). N, nucleus. X 75,000.
FIGS. 10 AND 11. Fig. 10 shows mouse myeloma cells (RPC 5.4) incubated for 60 min at 37°C in CF. CF is present in several stacked Golgi cisternae (arrows) and in small vesicles (ve) located near the Golgi cisternae or near lysosomes (ly). The two large lysosomes present in the field also contain considerable CF. Both lysosomes are surrounded by small vesicles or tubules that contain CF. Fig. 11 shows several such tubules (arrows) in continuity with another lysosome (ly), which is filled with CF. A virus particle is also present within this lysosome. × 75,000.
**Experiments with both CF and HRP.** In experiments in which cells were incubated with both tracers, most incoming vesicles were seen to contain both CF and HRP, but some contained CF alone. Surprisingly, after incubation in both tracers for 1 h at 37°C, there was a distinct difference in the distribution of these two tracers within cell compartments: Both CF and HRP were present together within lysosomes, but whereas CF was seen in the stacked Golgi cisternae, HRP was never observed in this location (Fig. 13). CF was also found in small vacuoles and tubular structures in the vicinity of lysosomes and the Golgi cisternae, many of which lacked HRP. Thus, the eventual destination of each tracer after a 1-h incubation was the same in the double-tracer experiments as when the cells were incubated in each tracer alone. These results indicate that the cell is somehow able to sort the two tracers in transit and to direct them to different compartments.

**Experiments with native (anionic) ferritin.** In contrast to the findings with CF, native ferritin did not bind to the surfaces of myeloma cells, and very small amounts were taken up even after prolonged incubation (up to 6 h) at very high concentrations of ferritin (5–10 mg/ml). In addition, the distribution of this tracer was limited to
lysosomes where it was found in only small amounts. It was never seen in the stacked Golgi cisternae.

**Experiments with ferritins with varying pI.** When cells were incubated with CF (prepared as described elsewhere \[20\]) with PI intermediate between that of native ferritin (4.8) and that of the commercially available CF (>8.0), fractions with a PI of 7.4 or less behaved like native ferritin—they did not bind to the cell surface and they were taken up in very small amounts into lysosomes only. Those with a PI of 7.8 or more behaved like the commercial CF—they bound to the cell surface, were taken up in significantly greater amounts, and, after a 1-h incubation, were found not only in lysosomes, but also in elements of the secretory apparatus—i.e., stacked Golgi cisternae and associated vesicles and vacuoles. These results were very instructive because they indicate that the intracellular fate of ferritin depends upon whether or not it binds to the cell membrane and acts as a membrane or a content marker. Anionic ferritin does not bind to myeloma or other cell membranes (because they are also negatively charged), enters the cell in the fluid content of endocytic vesicles (fluid-phase pinocytosis \[30\]), and ultimately appears only in lysosomes. CF (pI >7.8) binds by ionic interaction \(31\) to myeloma and other cell membranes, enters the cell bound to the inner aspect of the endocytic vesicle membrane (adsorptive pinocytosis \[30\]), and eventually appears both in the secretory and lysosomal compartments. The fate of ferritin with an intermediate PI depends upon whether under the prevailing conditions (cell surface charge, ionic strength of the medium, and PI of the tracer) it binds to the cell membrane, i.e., whether it serves as a membrane or a content marker.

**Immunocytochemical localization of immunoglobulins.** Intracellular immunoglobulins (IgG) were localized in RPC 5.4 and X63 Ag 8 myeloma cells by immunocytochemistry with peroxidase-conjugated anti-mouse Fab on cryostat sections. IgG were regularly stained throughout the rough ER, including the perinuclear cisterna, throughout most of the stacked Golgi cisternae, and in a few large vacuoles in the Golgi region (Fig. 14). Staining of the ER was present even in areas in which many intracisternal virus particles were located. In experiments in which immunocytochemical localization of IgG was carried out after exposure to CF, both the tracer and the IgG could be demonstrated in the same Golgi cisternae (Figs. 15 and 16). Thus, it was clear that, as in the case of plasma cells, the CF reached compartments along the secretory pathway.

**Comments on the consistency of immunocytochemical staining.** The advantage of using cryostat sections was that the cells, and thereby the main secretory compartments, are cut open (eliminating the necessity for using detergents) and thus directly exposed to either HRP or the labeled antibody fragment. It was clear that the use of cryostat sections greatly facilitated the penetration of the immunoreagents because little or no intracellular staining was seen when whole (unsectioned) cells were used. The consistency with which immunostaining was seen in a given compartment depended upon its extensiveness and, therefore, the frequency with which it was cut open by cryostat sectioning. Thus, the rough ER (including the perinuclear cisterna), being the most extensive and uniformly distributed compartment, was regularly exposed and hence regularly stained in all cells. The Golgi apparatus, whose distribution is more restricted, was less regularly cut open and, accordingly, less regularly stained. In myeloma cells, transitional vesicles (Figs. 4 and 14) that presumably contain secretory product were usually not stained because, owing to their small
Figs. 14–16. Immunocytochemical demonstration of IgG in myeloma cells (RPC 5.4). In Fig. 14 the secretory product (IgG) is present in the rough ER (er) including the perinuclear cisterna (pc), and in Golgi elements. All the stacked Golgi cisternae (G) are stained. A large vacuole (v) in the Golgi region also contains demonstrable IgG. N, nucleus; m, mitochondria. Figs. 15 and 16 are high magnification views of a cell incubated in CF for 60 min before fixation and the immunocytochemical localization of IgG. An area with relatively weak staining of the Golgi was chosen for illustration so that the CF shows up more clearly. It can be seen that CF (short arrow) is present in the same cisterna which contains IgG (long arrow). CF is also located in numerous vesicles (ve) located in close proximity to the Golgi cisternae (G). Fig. 16 is a further enlargement of part of Fig. 15 demonstrating both CF and IgG in Golgi cisternae. Specimen preparation as for Fig. 5 except that the cryostat section was incubated in peroxidase-conjugated anti-mouse IgG before staining in DAB. Fig. 14: × 32,000. Fig. 15: × 57,000. Fig. 16: × 100,000.
size, they were rarely cut open, and the labeled antibody fragment was apparently
unable to penetrate their membrane under the conditions used. It is of interest that
staining of the small Golgi-associated vesicles and of the Golgi cisternae was less
uniform and consistent than in plasma cells incubated in HRP. This could reflect the
fact that the labeled antibody fragment, which must diffuse into the myeloma cells to
achieve staining, is larger (80,000 mol wt) (19) than the HRP (40,000 mol wt) used as
a probe in the case of the plasma cells.

Localization of Aryl Sulfatase. Tests for this lysosomal marker enzyme were
carried out on RPC 5.4 myeloma cells to confirm the identity of lysosomal structures. 2
Reaction product for aryl sulfatase was restricted to typical lysosomal structures
(dense bodies, multivesicular bodies, or vacuolar structures), which could usually be
found in the Golgi region. Reaction product was not seen in the stacked Golgi
cisternae or in other Golgi-associated structures in these cells.

Discussion

The main finding in this study is the demonstration that in immunoglobulin-
secreting cells, membrane is continually retrieved from the cell surface and fuses with
Golgi elements. We have demonstrated the existence of considerable membrane traffic
from the cell surface to Golgi cisternae and secretory vacuoles as well as to lysosomes
both in plasma cells obtained from lymph nodes and in cultured myeloma cells. The
variations in membrane traffic and the pathways revealed with electron-dense tracers
are quite comparable to those previously detected in anterior pituitary cells (15, 16):
all the tracers used were taken up by endocytosis, but those that mark the contents of
endocytic vesicles (anionic ferritin and HRP) reached only lysosomes, whereas those
that mark the membrane (CF) reached secretory compartments as well as lysosomes.
Similar findings have also been reported in the thyroid gland (17). The most plausible
explanation for this membrane traffic to the Golgi in immunoglobulin-secreting cells
as well as in the other cell types mentioned is that it is connected with the recycling
of secretory vacuole membrane-i.e., membrane added to the cell surface by exocytosis
is assumed to be recovered by endocytosis and returned to the Golgi complex where
it is reused as containers in the packaging of newly synthesized immunoglobulins. 3
This assumption is supported by the fact that CF becomes located in compartments
that also contain immunoglobulins (demonstrated by immunostaining) of cells known
to be actively secreting proteins into the medium.

CF is not an ideal membrane marker because its binding to membranes is based
primarily on charge (electrostatic) interaction, and thus the tracer is susceptible to
detachment if the vesicle fuses with a compartment in which there is competition for
binding with other acidic groups of higher charge density than those on the membrane.
For this reason, in this as in previous work with CF, we cannot be certain that the
membrane removed from the cell surface is the same membrane as that that ultimately

2 Tests for acid phosphatase (with β-glycerophosphate or cytidine monophosphate as substrate) were
also done, but this enzyme was not a reliable lysosomal marker because it stained the cell membrane and
Golgi cisternae as well as lysosomes in these cells.

3 So far, we have been unable to obtain clear evidence for release of new secretory vesicles that contain
CF because of difficulty in removing residual CF bound to the cell surface. This is because (as reported
earlier [15]), upon addition of CF, the cells stick together with a monomolecular layer of CF in between,
which we have not been able to entirely eliminate even after repeated washing with buffers of high ionic
strength in the presence of heparin.
appears marked with CF in the Golgi. Be that as it may, the fact is that because of its interaction (albeit weak) with the membrane, CF has revealed a membrane traffic in immunoglobulin-secreting cells and in several other cell types that had not previously been detected. To obtain more information on membrane recycling in secretory cells, what is needed at present are (a) stable (covalently bound), nonperturbing membrane labels, and (b) systems amenable to their use and analysis. Our results indicate that appropriately selected myeloma cell lines may represent suitable and promising systems in which to investigate membrane recycling in further detail because, (a) we have demonstrated the existence of membrane traffic in immunoglobulin-secreting cells similar to that in glandular cells, and (b) we have identified several myeloma cell lines that behave like normal plasma cells with respect to these properties. In particular, the availability of a homogeneous cell population that can be propagated in culture in large quantities makes this a more feasible system on which to carry out cell fractionation and subsequent biochemical studies than the lobule (14, 17) or dissociated cell (15) systems used previously.

There is now abundant evidence (1–5) that plasma cells use the same general pathway in the production of immunoglobulins as that used by exocrine and endocrine cells for production of their secretory products. Indeed, the only difference reported between plasma cells (as well as a few other cell types such as fibroblasts [6, 32]) and other secretory systems is the fact that secretion is continuous, and storage and release of immunoglobulins are unaffected by many of the known control mechanisms (e.g., cyclic nucleotides and extracellular Ca2+). Our findings provide evidence for yet another similarity between plasma cells and other secretory cells—i.e., the recovery and handling of surface membrane. It should be mentioned, however, that recently Cohen et al. (33) have hypothesized that there is undirectional membrane flow in myeloma cells and that manufactured membrane is rapidly degraded. This conclusion was based on data obtained on myeloma cells (MOPC 41), which indicate that a number of proteins of a microsomal membrane fraction (consisting of a heterogeneous mixture of ER, Golgi, and plasma membranes) turn over at the same rate. However, the validity of this conclusion can be questioned for a number of reasons: (a) the fractions examined were heterogeneous, (b) the possibility that the data reflect the turnover of viral membrane proteins (which are concentrated in the ER) was not considered, and (c) the fact that these myeloma cells are rapidly dividing and in a state of exponential growth (rather than steady-state) complicates the interpretation of the data. There is now reliable data obtained under steady-state conditions, on several other secretory systems, which indicate that the membranes of secretory granules turnover at a much slower rate than that of their content. Thus, the recent turnover data (34, 35), as well as the data obtained with electron-dense tracers (14–18), suggest that granule membrane released during exocytosis is reused or recycled rather than being degraded. Other studies, notably those by Steinman et al. (36, 37), and Schneider et al. (38) provide evidence for the existence in cultured cells (macrophages and fibroblasts) of recycling (plasmalemma → lysosomes → plasmalemma) of membrane internalized by pinocytosis (36, 38) and phagocytosis (37).

An interesting and novel finding obtained in this study was the demonstration that when a membrane marker (CF) and a content marker (HRP) are added to the same
cell suspension, the tracers are taken up (for the most part⁴) contained within the same endocytic vesicles, but they maintain their different destinations. These results could be explained if the incoming endocytic vesicles first fuse with lysosomes where they lose their content (containing HRP and any loosely bound or free ferritin) and the membrane with bound CF moves on to the Golgi. This indirect route has the advantage that it provides a means of preventing exogenous proteins from reaching the secretory compartment.

In previous work from this laboratory on anterior pituitary cells (15), we made note of the fact that the variations in traffic detected with anionic and cationic ferritin could be explained either by the existence of two recovery routes (plasmalemma → Golgi, and plasmalemma → lysosomes), or by a single recovery route with two stations, the lysosomes being the first station and the Golgi being the second. The data obtained did not allow us to distinguish between these two possibilities. In the meantime, Herzog and Miller (17) have obtained data that indicate the existence of such an indirect Golgi route (plasmalemma → lysosomes → Golgi) in thyroid cells. On the other hand, studies on exocrine cells of the parotid (14) and the pancreas (18) indicate that membrane recovery is rapid and extensive with little evidence for a lysosomal stopover, which makes it likely that the direct route (plasmalemma → Golgi) is used extensively in these cells. Thus, the most likely possibility at present is that in secretory cells both routes are used, and individual cell types divert the traffic according to their activities or functional state.

Finally, a word should be said about the importance of the net charge of tracers used in the study of endocytosis and membrane internalization as exemplified by our experience with differently charged ferritins. Results were consistent with native ferritin, which, because of its net negative charge, does not bind to cell surfaces and acts strictly as a content marker. In all systems studied to date (13–16) this form of the tracer appears only in lysosomes. Similarly, results appear straight forward with commercially available CF that carries a high net positive charge, binds to most cell membranes (15, 17, 31), is selectively concentrated during endocytosis, and acts, at least initially, as a membrane marker. A problem arises, however, with the use of ferritins with a pI near neutrality (7.4–7.8): they bind to cell membranes to a variable degree depending upon such factors as the concentration of negatively charged sites on the cell type in question and the ionic strength of the medium in which exposure takes place. This variability in binding results in variability in subsequent intracellular distribution of the tracer. It seems likely that this same situation could arise with the use of other tracers or in using ferritin conjugates in which the charge has been modified during conjugation.

**Summary**

Evidence for recovery of surface membrane and its fusion with Golgi cisternae has been obtained previously in several glandular cells. This study was conducted to determine whether or not membrane is similarly retrieved from the surfaces of plasma cells from lymph nodes (of rats immunized with horseradish peroxidase [HRP]) and

⁴ As mentioned in the Results, a few vesicles near the cell surface contain CF alone. It cannot be determined whether they are from regions of the free cell surface where HRP has access or from regions where HRP is excluded—i.e., those regions in which cells are in contact with a monomolecular layer of CF in between (see ⁴).
mouse myeloma cells (RPC 5.4 and X63 Ag 8 cell lines). Electron-dense tracers (cationic and anionic ferritin, HRP) were used to trace the pathways followed by surface membrane recovered by endocytosis, and immunocytochemistry was used to identify the secretory compartments.

When plasma cells or myeloma cells were incubated with cationized ferritin (CF), it bound to the cell surfaces and was taken up in endocytic vesicles, for the most part bound to the vesicle membrane. After 30–60 min, it was found increasingly within lysosomes and in several secretory compartments—notably in multiple stacked Golgi cisternae and secretory vacuoles. By immunocytochemistry the secretory product (immunoglobulins) and CF could be demonstrated in the same Golgi components. When myeloma cells were incubated with native (anionic) ferritin or in HRP, these tracers were taken up in much smaller amounts, primarily within the contents of endocytic vesicles. With continued incubation, they appeared only in lysosomes. When cells were doubly incubated, first in CF and then in HRP, both tracers were taken up (often within the same endocytic vesicle), but they maintained their same destinations as when incubated in a single tracer alone: the content marker, HRP, was localized exclusively within the lysosomal system, whereas the membrane marker, CF, was found within elements along the secretory pathway as well as within lysosomes.

The findings demonstrate the existence of considerable membrane traffic between the cell membrane and the Golgi cisternae and lysosomes in both normal plasma cells and myeloma cells. Because myeloma cells behave like the glandular cells studied previously with regard to pathways of retrieved surface membrane, they represent a suitable and promising system for further studies of mechanisms and pathways of membrane retrieval and recycling in secretory cells.

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