

The Rough Endoplasmic Reticulum and the Golgi Apparatus Visualized Using Specific Antibodies in Normal and Tumoral Prolactin Cells in Culture

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ABSTRACT Antibodies directed against membrane components of dog pancreas rough endoplasmic reticulum (A-RER) and rat liver Golgi apparatus (A-Golgi) (Louvard, D., H. Reggio, and G. Warren, 1982, *J. Cell Biol.* 92:92-107) have been applied to cultured rat prolactin (PRL) cells, either normal cells in primary cultures, or clonal GH₃ cells. In normal PRL cells, the A-RER stained the membranes of the perinuclear cisternae as well as those of many parallel RER cisternae. The A-Golgi stained part of the Golgi membranes. In the stacks it stained the medial saccules and, with a decreasing intensity, the saccules of the trans side, as well as, in some cells, a linear cisterna in the center of the Golgi zone. It also stained the membrane of many small vesicles as well as that of lysosomelike structures in all cells. In contrast, it never stained the secretory granule membrane, except at the level of very few segregating granules on the trans face of the Golgi zone. In GH₃ cells the A-RER stained the membrane of the perinuclear cisternae, as well as that of short discontinuous flat cisternae. The A-Golgi stained the same components of the Golgi zone as in normal PRL cells. In some cells of both types the A-Golgi also stained discontinuous patches on the plasma membrane and small vesicles fusing with the plasma membrane.

Immunostaining of Golgi membranes revealed modifications of membrane flow in relation to either acute stimulation of PRL release by thyroliberin or inhibition of basal secretion by monensin.

The involvement of the rough endoplasmic reticulum (RER) and the Golgi apparatus in the secretory pathway of prolactin (PRL) was originally proposed by Smith and Farquhar (17) on the basis of Palade's concept of the secretory process (13). This was first suggested by the ultrastructural organization of rat PRL cells in the lactating rat (17) and further supported by quantitative autoradiographic analysis of pulse-chase experiments performed on prolactin cells (5, 16, 19). Most recently, immunocytochemistry has permitted the detection of the secretory product within both membrane compartments in normal prolactin cells in primary culture (22), as well as in the tumoral prolactin cell line GH₃ (23, 24). In the latter cell type, electron microscopic studies have been used to follow the ultrastructural modifications resulting from stimulation of hormonal release

by a specific ligand, the hypothalamic tripeptide "TRH" (thyrotropin-releasing hormone). Such modifications included an extension of the Golgi zone with a proliferation of smooth vesicles in the Golgi region and beneath the plasma membrane (8, 20).

Data concerning the biochemistry of RER membranes and Golgi membranes are still lacking in PRL cells as well as in many other endocrine cells. Since specific antibodies directed against membrane components of dog pancreas RER and rat liver Golgi apparatus were very recently obtained by Louvard et al. (10), it was of great interest to apply these antibodies to rat prolactin cells. Such immunological tools would moreover permit one to follow modifications induced in the organization of these compartments in relation to stimulation of prolactin

secretion.

MATERIALS AND METHODS

Cell Cultures

PRIMARY CULTURES: Cells were obtained by enzymatic dispersion of normal male Wistar rat (200-g weight) anterior pituitaries as previously described (22). For experiments, 4×10^5 cells were plated per 35-mm plastic tissue culture dish (Falcon, Becton Dickinson, Grenoble, France) in 2 ml of culture medium composed as follows: Ham's F10 solution supplemented with charcoal-dextran-treated (3) horse serum (10%) and fetal calf serum (2.5%), antibiotics, and 4×10^{-8} M 17 β -estradiol. Cultures were maintained at 37°C in 5% CO₂ in air, and medium was renewed after 4 d and then after 2 d. Cultures were used after 7 d.

GH₃ CELLS: Experiments were performed with GH₃/B₆ cells, a subclone of GH₃ cells (7). The cells are routinely grown in Ham's F10 medium supplemented with 15% horse serum and 2.5% fetal calf serum. For experiments, 1×10^5 cells were plated per 35-mm culture dish in 2 ml of culture medium and were grown at 37°C in 5% CO₂ in air for 6 d. Experiments were performed 20–24 h after the last medium change.

Cell Treatment

Cultures were rinsed with previously warmed F10 solution and then incubated at 37°C in a controlled atmosphere (5% CO₂ in air) with 1 ml of F10 supplemented or not with 27 nM synthetic TRH (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA) for 30 min to 4 h (primary cultures) or for 1 h (GH₃ cells).

Some dishes were preincubated for 15 min in 1 ml of F10 supplemented with 1 μ M monensin (sodium-salt, Calbiochem-Behring Corp.) and then incubated in 1 ml of F10 supplemented with 1 μ M monensin in the presence or absence of 27 nM TRH.

At the end of incubation periods, the medium was collected for radioimmunoassay of PRL as previously described (24). Cells were rapidly rinsed with F10 medium and fixed in situ.

Immunocytochemical Procedure

Primary cultures were fixed in situ with 0.4% glutaraldehyde in 0.01 M phosphate-buffered saline (PBS) with 2 mM MgCl₂ and 0.08M NaCl, pH 7.4, according to Ohtsuki et al. (12) for 15 min at 4°C as previously described (22). GH₃ cells were fixed in situ with a mixture of 2% formaldehyde, 0.05% glutaraldehyde in Sorensen buffer for 1 h at 4°C as previously described (24). After washing in the same buffer, cells were permeabilized with 0.05% saponin (primary cultures) or with 0.03% saponin (GH₃ cells) in 0.01 M PBS at room temperature for 30 min. The immunocytochemical staining was performed directly in the culture dishes as previously described (22, 24), using the indirect immunoperoxidase technique. Saponin (0.05% or 0.03%) was added to antisera and rinsing solutions. Cells were incubated in immunoglobulin fractions purified from specific immunsera raised in rabbits against Golgi and RER membranes (0.1 mg/ml and 0.2 mg/ml, respectively) for 1½ h at room temperature, washed in PBS for 30 min, and incubated with peroxidase-labeled sheep antibodies against rabbit immunoglobulins (Institut Pasteur, Paris) for 1½ h at room temperature. After washing, cells were fixed with 1% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.4, for 30 min at 4°C and washed in the same buffer. After the peroxidase

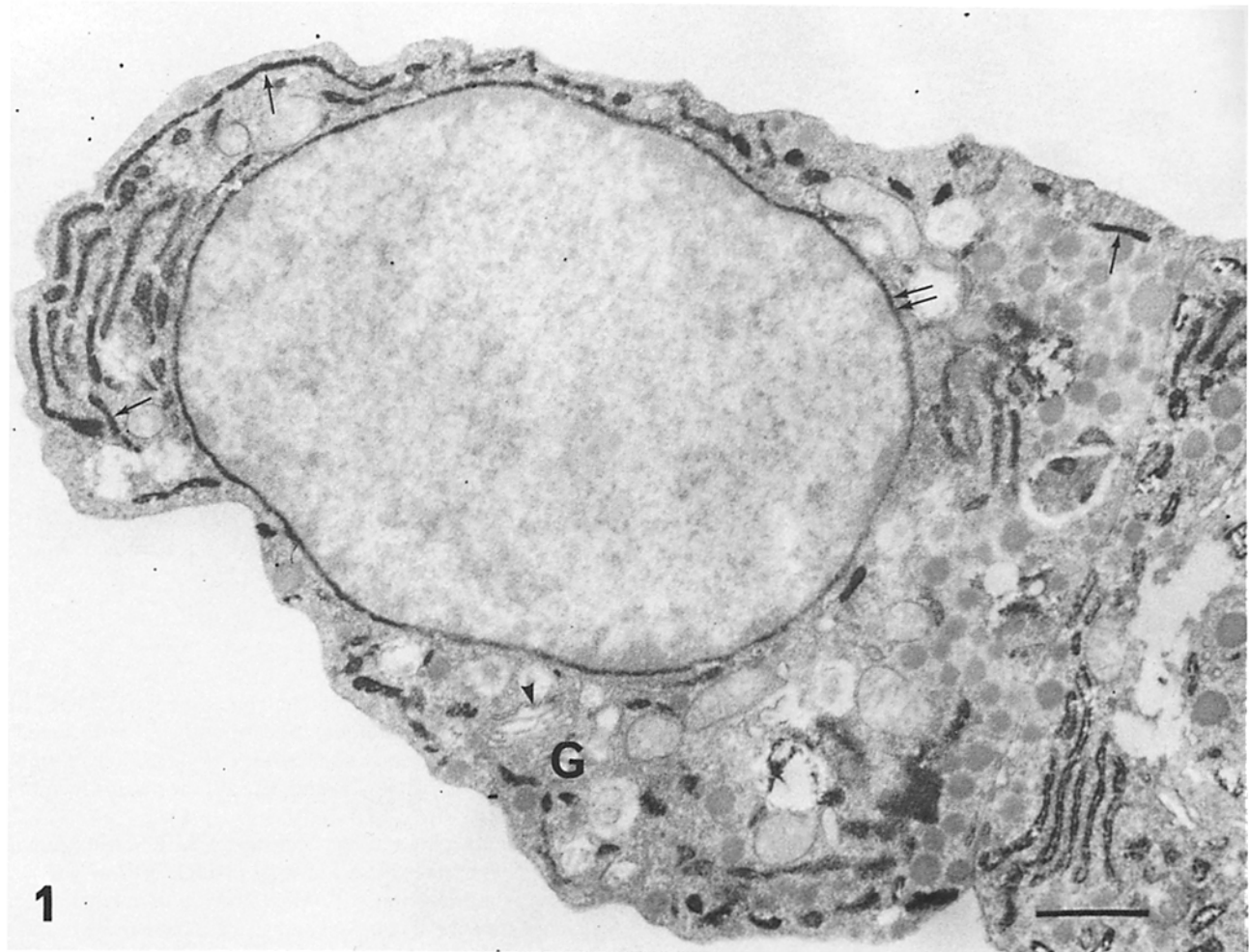


FIGURE 1 A normal prolactin cell in primary culture immunocytochemically stained with anti-RER antibodies. The anti-RER antibodies labeled parallel rows of linear flattened cisternae (arrows) as well as the perinuclear cisterna (double arrows). The plasma membrane, the membrane of secretory granules, and the smooth saccules of the Golgi apparatus (G) were completely free of reaction product. The arrowhead indicates a possible transitional element between RER and Golgi saccules which is slightly stained. Bar, 1 μ m. $\times 15,000$.

visualization according to Graham and Karnovsky (9), cells were postfixed in 1% osmium tetroxide, dehydrated, and embedded in situ in Epon according to Brinkley et al. (2). Immunoreactive cells were located under the light microscope and photographed. Selected zones were cut with a warm punch and mounted on Araldite blocks, as previously described (15), and sectioned in a horizontal direction. Ultrathin sections were examined by electron microscopy without further staining.

Antibodies to RER and Golgi Complex

The preparation of antibodies to microsomal and Golgi fractions was described in detail by Louvard et al. (10). The antibodies were obtained in rabbits immunized with purified membrane fractions of rough microsomes from dog pancreas or Golgi vesicles from rat liver. Unwanted antibodies raised to components other than those unique to either RER or the Golgi complex were removed by suitable absorption steps. The purified antibodies to Golgi membranes were found to be directed almost entirely against a single polypeptide with an apparent molecular weight of 135,000 and the purified antibodies to RER were directed against four polypeptides with molecular weights of 29,000, 58,000, 66,000, and 91,000 (10).

RESULTS

IMMUNOCYTOCHEMICAL VISUALIZATION OF THE RER AND GOLGI APPARATUS

Primary Cultures

ANTIBODIES SPECIFIC FOR THE RER: At the light microscopic level, these antibodies labeled numerous linear structures present in the cytoplasm of glandular cells and of fibroblasts in primary cultures. Moreover, the periphery of the nucleus was well delineated by the brown reaction product.

At the electron microscopic level, prolactin cells, with large and polymorphous secretory granules, were easy to identify in the small clusters of glandular cells. The anti-RER antibodies labeled parallel rows of linear, flattened cisternae as well as the perinuclear cisternae (Fig. 1). Ribosomes were not stained and therefore were difficult to identify on the outer face of the membrane of these cisternae. Nevertheless, the distribution of

the stained cisternae corresponds very well to the organization of the rough endoplasmic cisternae as previously identified by conventional electron microscopy (17). It was difficult to determine on which face of the membrane the reaction product was located. Other cellular structures, particularly the plasma membrane, the membrane of secretory granules, and the stacks of the smooth saccules of the Golgi zone, were completely free of reaction product (Fig. 1).

ANTIBODIES SPECIFIC FOR THE GOLGI APPARATUS:

Basal Conditions of PRL Secretion: At the light microscopic level, these antibodies labeled numerous small spots scattered in the cytoplasm of the cells but were more abundant in a large area near the nucleus. The labeling was more intensive in glandular cells than in flattened fibroblasts (Fig. 2).

At the electron microscopic level, the anti-Golgi antibodies clearly labeled smooth membranes of the Golgi zone in glandular cells. In PRL cells, most but not all of the Golgi saccules were labeled in stacks. In general, the staining intensity was maximum on medial saccules and progressively decreased on the saccules of the trans side (Fig. 3). In some cells, a few linear cisternae were labeled in the core of the Golgi zone. In addition, many but not all vesicles were stained (Fig. 3). The reaction product appeared to be attached to the inner face of the membrane, in these structures. A positive reaction was also observed on peripheral as well as inner membranes of lysosomal-like structures, and within some multivesicular bodies. In contrast, the membranes of secretory granules were never stained except for membranes of a very few segregating secretory granules (Fig. 4). In some cells, discrete areas of the plasma membrane were underlined with reaction product and a small number of small vesicles beneath the plasma membrane could be seen.

The rough endoplasmic reticulum including the nuclear

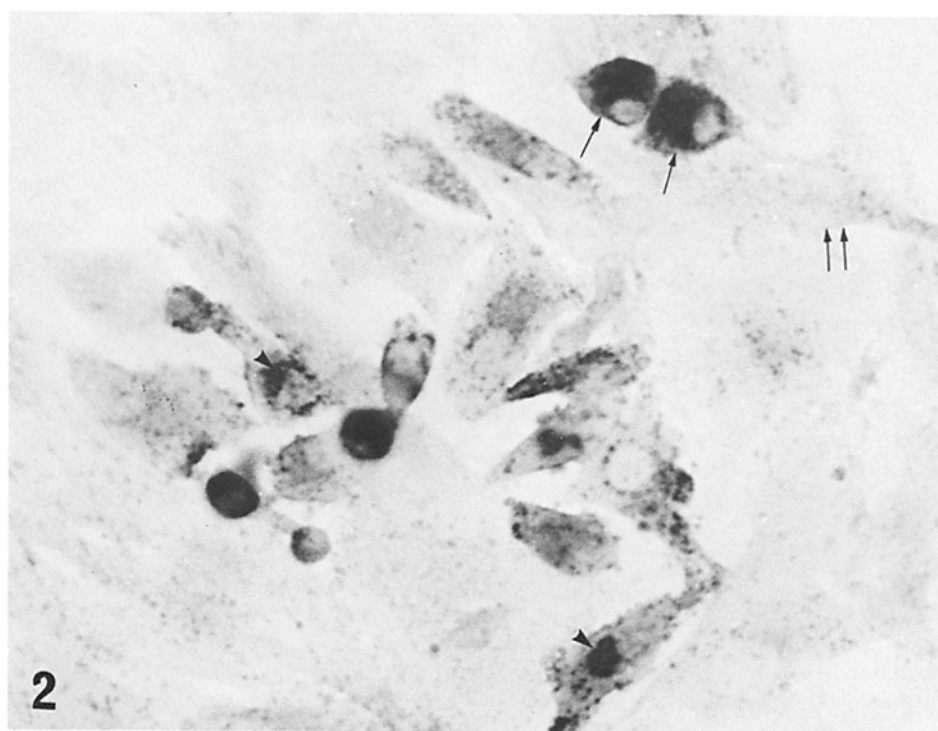


FIGURE 2 A primary culture immunochemically stained with anti-Golgi antibodies. These antibodies labeled numerous small spots scattered in the cytoplasm of the cells but more abundant in a large area (arrowheads) near the nucleus. The labeling was more intensive in glandular cells (arrows) than in flattened fibroblasts (double arrows). $\times 600$.

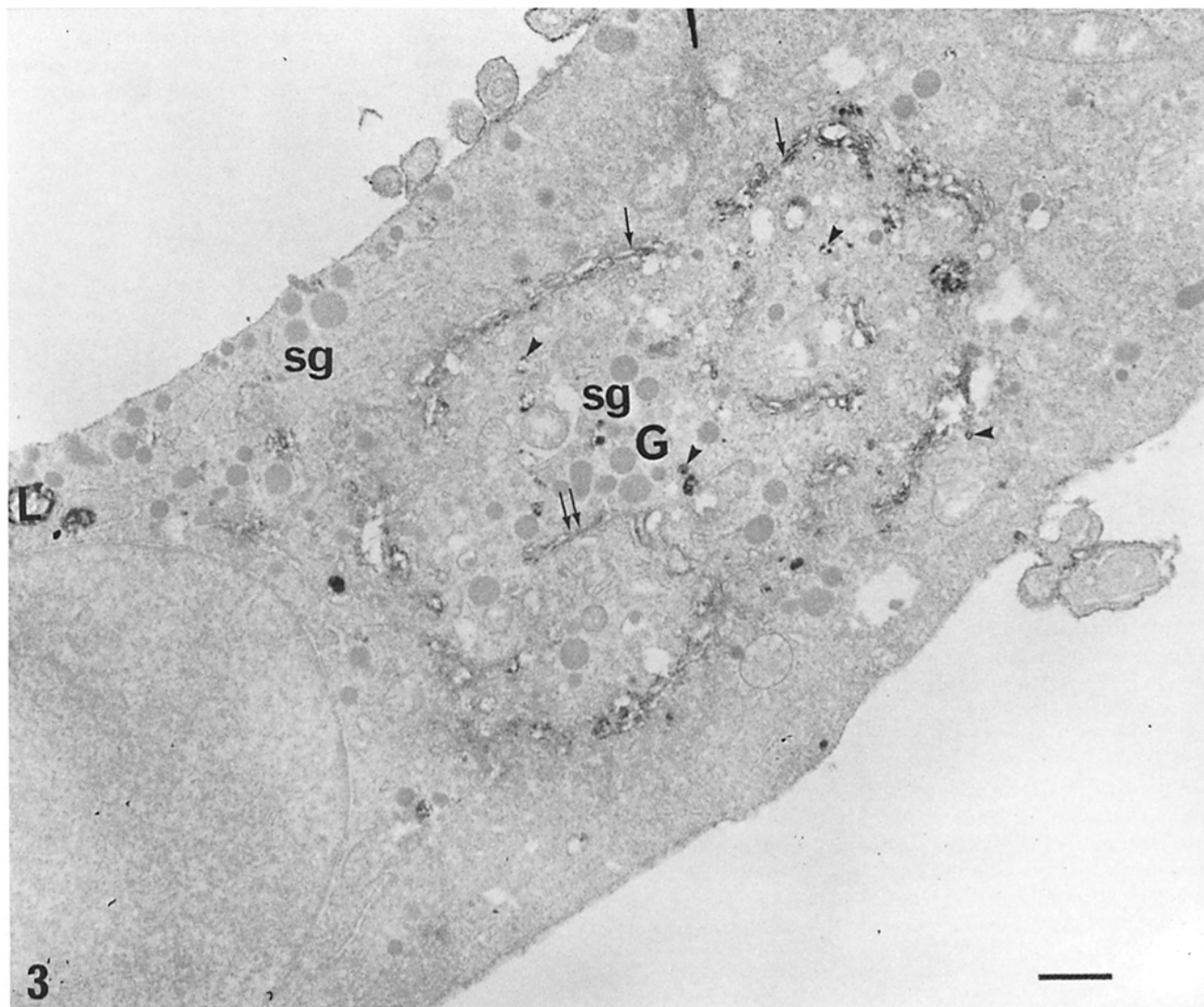


FIGURE 3 A normal prolactin cell immunochemically stained with anti-Golgi antibodies. The anti-Golgi antibodies labeled the smooth membrane of most but not all of the saccules in the Golgi zone (G). The staining intensity was maximum on medial saccules (arrows) and progressively decreased on the saccules of the trans side. Some cisternae (double arrows) were labeled in the core of the Golgi zone as well as many but not all vesicles in this region (arrowheads). A positive reaction was also observed on peripheral as well as inner membranes of lysosomelike structures (L). In contrast the membrane of secretory granules (sg) was not stained. The RER cisternae including the perinuclear cisterna were unlabeled. Bar, 1 μm . $\times 10,000$.

membrane was not labeled, nor were the mitochondria or the nuclear matrix. In control experiments using normal rabbit immunoglobulins, there was no labeling of any cellular structures.

Effects of TRH or Monensin: The stimulation of PRL secretion by TRH or its inhibition by monensin have been assayed by PRL radioimmunoassay (data not shown). In some cells TRH treatment induced an extension of the Golgi zone within 1 h. The most striking effect consisted of an increased number of immunoreactive small vesicles in the Golgi zone, as well as in the cytoplasm and beneath the plasma membrane (Fig. 4). In these stimulated cells the plasma membrane was dotted with numerous patches of reaction product (Fig. 4). After monensin treatment the anti-Golgi antibodies labeled the membrane of large and dilated vacuoles (see Fig. 9 a).

GH₃ Cells

ANTIBODIES SPECIFIC FOR THE RER: At the light microscopic level, these antibodies labeled some short elongated structures in the cytoplasm of GH₃ cells as well as the nuclear periphery (Fig. 5 a).

At the electron microscopic level, the anti-RER antibodies labeled short and discontinuous cisternae as well as the perinuclear cisternae (Fig. 6). The distribution of these cisternae corresponds very well to the organization of the RER of GH₃ cells, as already described using conventional electron microscopy (8). As in normal prolactin cells, the other cell structures were completely free of reaction product. There was no evident effect of TRH or monensin on the organization of the immunocytochemically labeled RER cisternae of GH₃ cells.

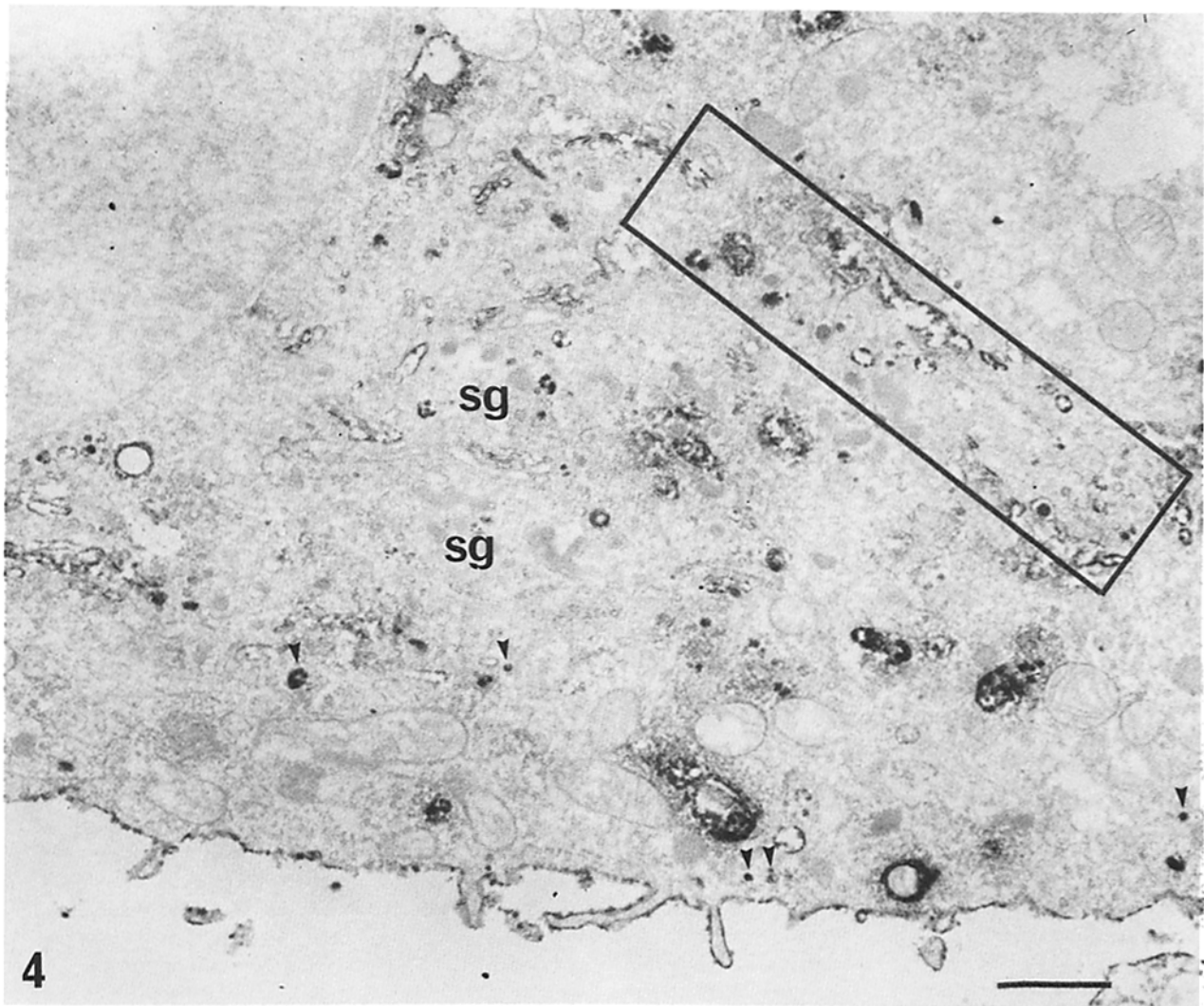
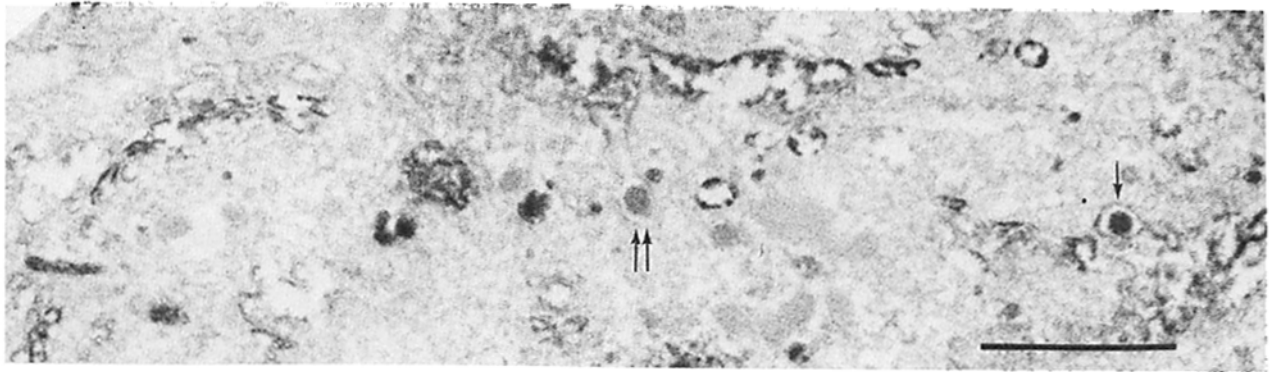


FIGURE 4 A normal prolactin cell exposed to TRH for 2 h immunochemically stained with anti-Golgi antibodies as in Fig. 3. In this cell, which contained a low amount of secretory granules (sg), TRH treatment induced an extension of the Golgi zone and an increased number of immunoreactive small vesicles in this zone as well as in the cytoplasm and beneath the plasma membrane (arrowheads). Moreover, the plasma membrane was punctuated by numerous patches of reaction product. Bar, 1 μm . $\times 16,000$. Inset: At a higher magnification, on the trans face of this Golgi zone, the membrane of segregating secretory material (arrow) was slightly underlined with reaction product whereas the membrane of an adjacent segregating secretory granule (double arrow) was not stained. Bar, 1 μm . $\times 26,000$.

ANTIBODIES SPECIFIC FOR THE GOLGI APPARATUS:

Basal Conditions of PRL Secretion: At the light microscopic level, these antibodies labeled, in GH_3 cells as in normal prolactin cells, numerous small spots scattered in the cytoplasm,

most abundantly near the nucleus (Fig. 5b).

At the electron microscopic level, the anti-Golgi antibodies labeled the Golgi zone of GH_3 cells, which is apparently composed of several units, at least in thin sections (8). In any

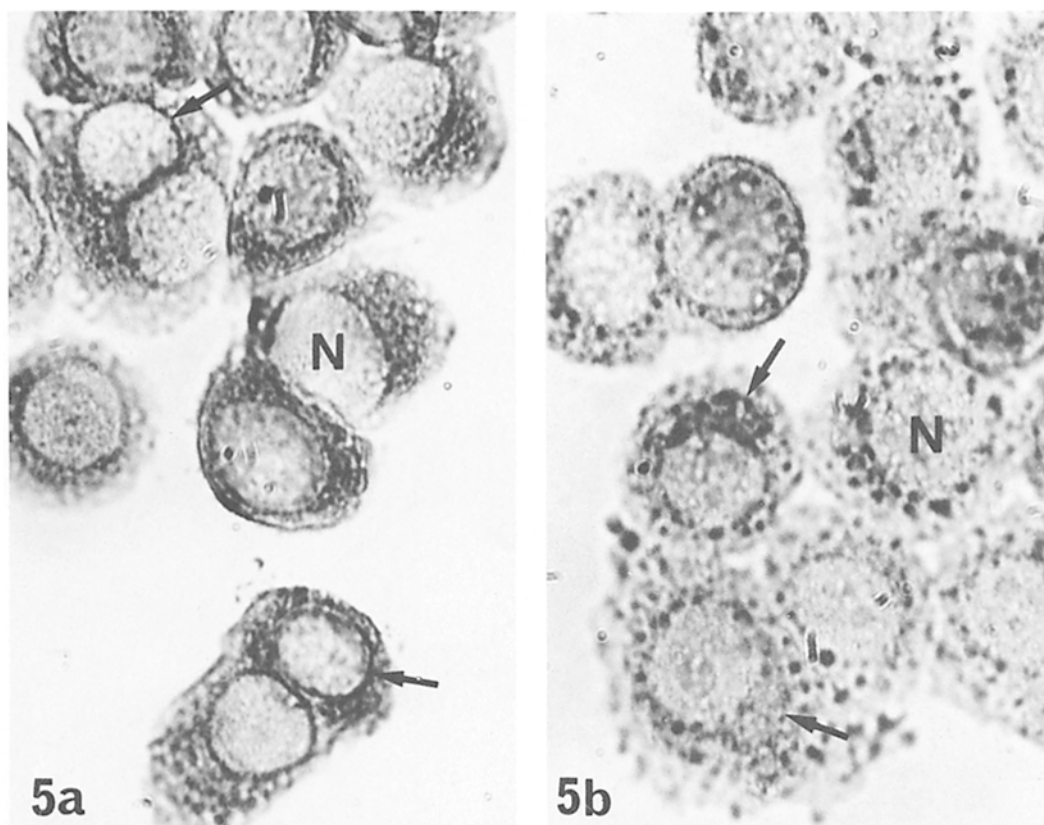


FIGURE 5 GH₃ cells immunochemically stained with anti-RER antibodies (a) and with anti-Golgi antibodies (b). (a) The anti-RER antibodies labeled clearly some short elongated structures in the cytoplasm as well as the nuclear membrane (arrows). N, nucleus. $\times 1,200$. (b) The anti-Golgi antibodies labeled numerous small spots scattered in the cytoplasm, but most abundantly in a large area (arrows) near the nucleus (N). $\times 1,200$.

Golgi unit the smooth membranes of most, but not all, Golgi saccules were labeled. The most intense staining was generally observed on the medial saccules, although this was less evident than in normal PRL cells. The trans and cis sides of the Golgi zone are difficult to define in GH₃ cells. In addition, the membranes of many of the small vesicles in this region were also stained (Fig. 7). Reaction product was also found in the same structures as in normal prolactin cells, i.e., on the membranes of small vesicles beneath the plasma membrane, and on the membranes of lysosomelike structures and of multivesicular bodies. In the two latter structures the reaction product was apparently localized on the inner face of the limiting membrane as well as on inner membrane whorls or vesicles.

Effects of TRH or Monensin: As reported for primary cultures the effects of TRH or monensin on PRL secretion have been controlled using PRL radioimmunoassay (25) (data not shown). Treatment with TRH induced modifications in the organization of the immunochemically labeled Golgi membranes. After 1 h, in many cells an extension of the Golgi zone was observed as previously seen by conventional electron microscopy (8). Concomitantly, the number of the immunoreactive small vesicles dispersed in the cytoplasm increased, particularly beneath the plasma membrane (Fig. 8a). Moreover, some of these vesicles were observed fusing with the plasma membrane (Fig. 8b).

In monensin-treated cells, the anti-Golgi antibodies labeled the membrane of numerous and large dilated vacuoles as well as some undilated elongated structures in the disorganized

Golgi region (Fig. 9b). Beneath the plasma membrane, some immunoreactive small vesicles or larger vacuoles were observed. Moreover, monensin did not prevent the increase in number of small immunoreactive vesicles induced by TRH treatment in the Golgi region and beneath the plasma membrane.

DISCUSSION

Antibodies directed against membrane components of dog pancreas RER and against those of rat liver Golgi apparatus have been applied for the first time to rat endocrine cells that secrete PRL. We have shown that they label the membrane of RER and part of the Golgi membranes in both normal and tumoral PRL cells. Such findings are in agreement with previous observations (10) indicating that the antibodies against RER label this structure in all cells tested, regardless of species, whereas the A-Golgi antibodies only label the Golgi zone in cells that originate from rodents. The labeling with each type of antibody is very specific as there is no evidence of overlap between the two membrane compartments that are stained. This specificity is the result of suitable absorption steps and has been shown to correspond to the recognition of distinct polypeptides (10). Therefore, the present findings provide direct evidence for a biochemical specificity of intracellular membranes in a secretory cell. The distribution of antigenic sites with respect to a membrane bilayer cannot be established using the immunoperoxidase technique described above and polyclonal antibodies.

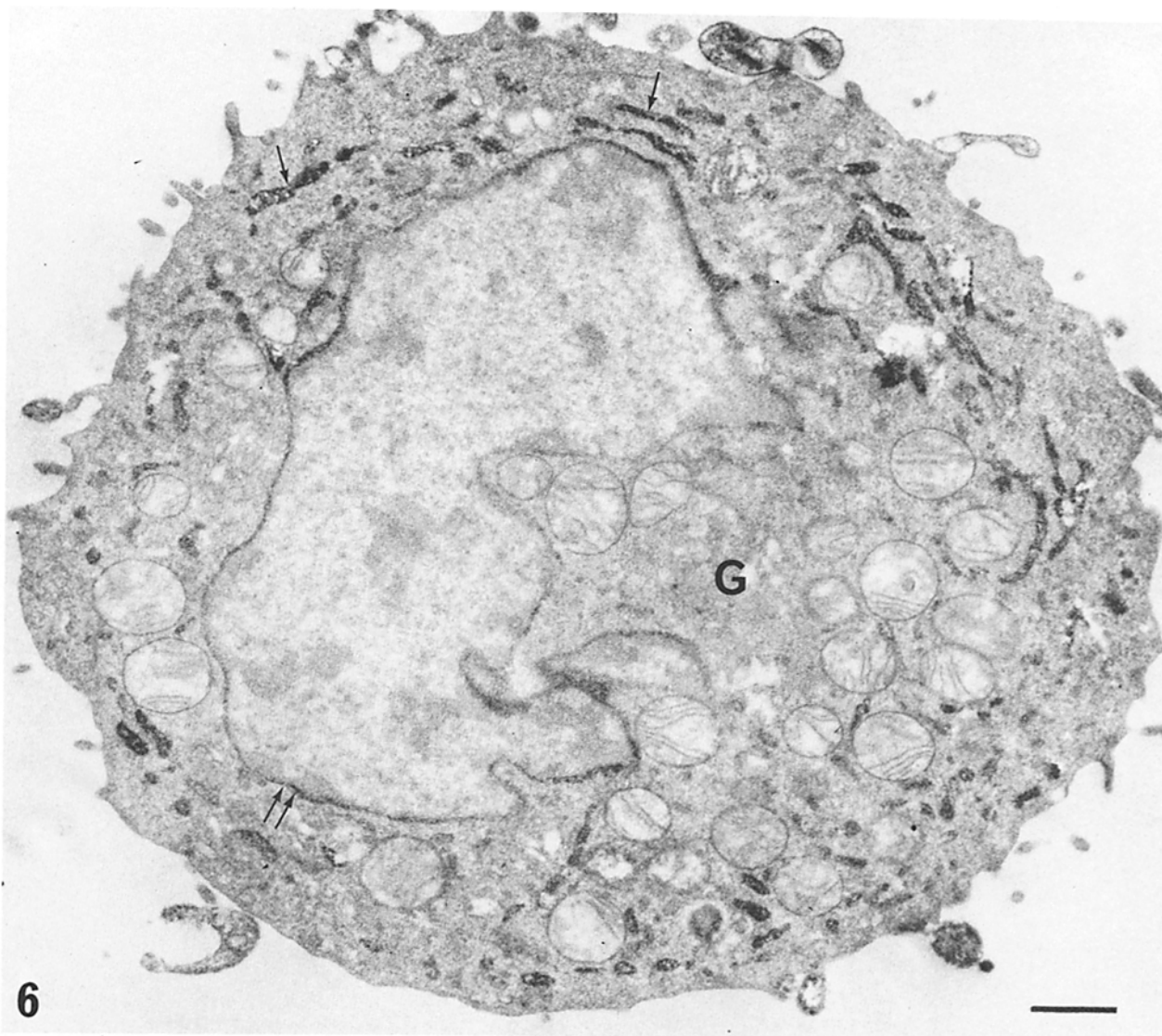


FIGURE 6 A GH₃ cell immunochemically stained with anti-RER antibodies. The anti RER antibodies labeled short and discontinuous cisternae (arrows) as well as the perinuclear cisterna (double arrows). The plasma membrane and the membrane of the smooth saccules of the Golgi apparatus (G) were completely free of reaction product. Bar, 1 μ m. \times 12,000.

Since normal PRL cells have often been proposed as a model for the transport and packaging of secretory proteins (4, 6), it is of interest to relate the present immunocytochemical findings to previous data obtained by other approaches on the same cell type. As concerns the RER, which displays very typical features in these cells, there is a complete agreement between pictures obtained by immunostaining of RER membranes, by conventional electron microscopy (14, 17), and by immunostaining of intracisternal PRL (22). All RER cisternae seemed to be visualized by the A-RER antibodies. The antigenic sites appeared to be localized preferentially on the inner face of the membranes. However, ribosomes were hard to identify because of the intensity of the peroxidase reaction. It was, therefore, difficult to determine precisely whether the transitional elements between RER and Golgi zone were labeled or not. Labeling of such elements as illustrated in Fig. 1 was very rare.

The Golgi zone of normal PRL cells has a typical appearance. As seen by conventional electron microscopy it is large,

rounded, and limited by several stacks of saccules. In its core, small granules arising within the inner Golgi cisternae aggregate to form mature secretory granules (17). The presence of PRL has been immunocytochemically revealed within the saccules as well as in masses of condensing secretory material, depending on the method used (22). Moreover, acid phosphatase activity has been cytochemically visualized within one or two of the transmost cisternae, as well as in an inner cisterna located a short distance from the trans face of the stacks which corresponds to the "GERL" as defined by Novikoff and Novikoff (11). In that cisterna as well as in the innermost saccule, the enzyme reaction product was often found associated with masses of condensing secretory material (17, 22). Compared to these data, it is clear that the A-Golgi antibodies labeled only part of the Golgi membranes. This can be related to the origin of these antibodies, which have been raised against a light Golgi fraction of rat liver that is believed to consist largely of secretory vacuoles from the trans side (1, 6). In view of the

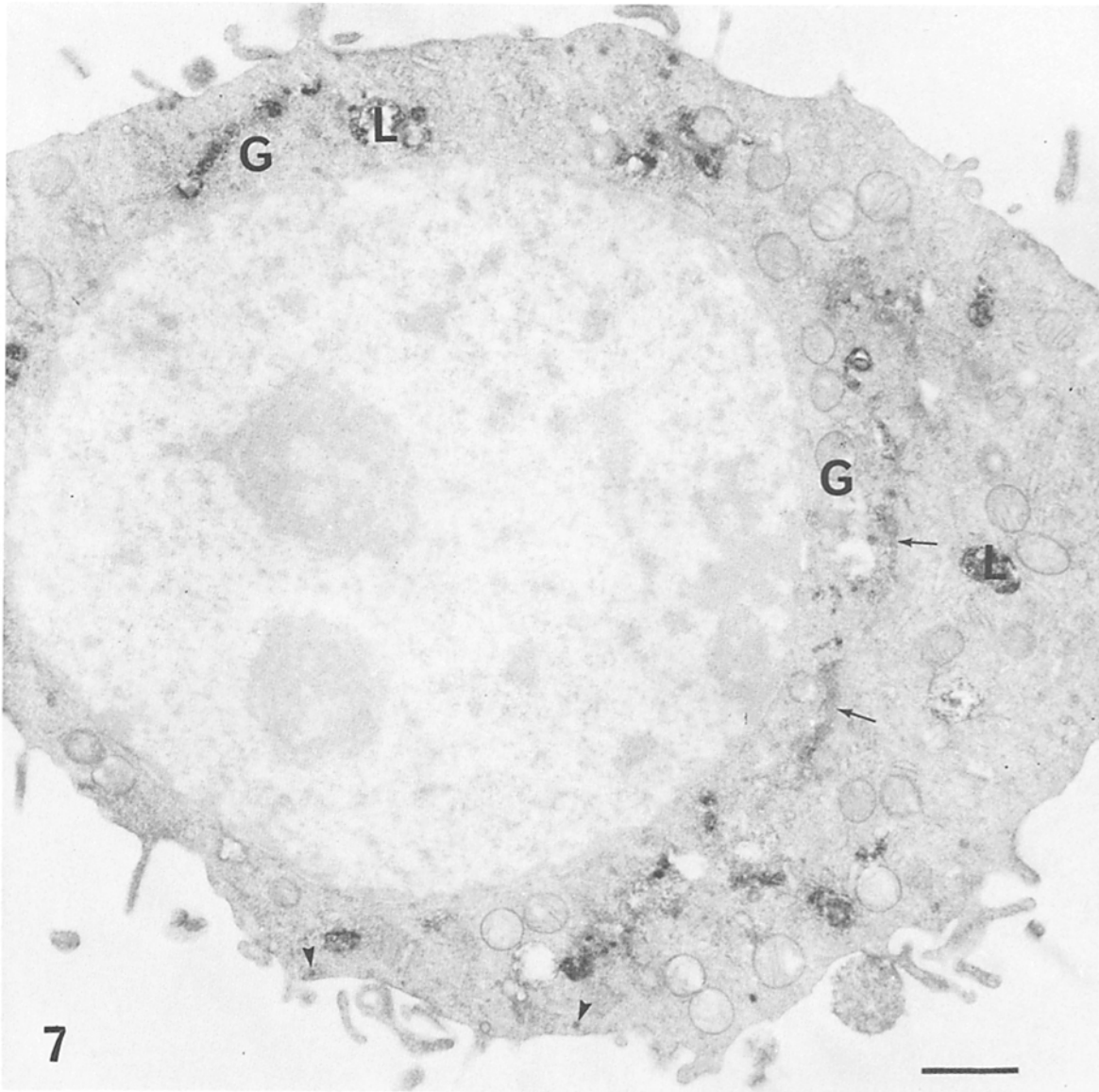


FIGURE 7 A GH₃ cell immunochemically stained with anti-Golgi antibodies. The anti-Golgi antibodies labeled the smooth membrane of most, but not all, saccules of the Golgi zone (G). The most intense staining was observed on the medial saccules (arrows). Reaction product was also found on many of the small vesicles in this region and beneath the plasma membrane (arrowheads). The membrane of lysosomelike structures (L) was labeled as well as their inner membrane whorls. Bar, 1 μ m. \times 13,500.

well-recognized heterogeneity of Golgi membranes and of the big differences in organization between liver cells and anterior-pituitary cells, such a partial labeling is not surprising. Of great interest, however, is the fact that the A-Golgi antibodies labeled several components of the Golgi zone or its derivatives: medial saccules of the trans face; a few linear cisternae in the core of the Golgi zone (in some cells); many small vesicles and lysosomes, which are particularly numerous in cultured PRL cells. The typical topography of the GERL cisternae was not well-preserved by our fixation and permeabilization conditions but a slight labeling of these membranes with A-Golgi antibodies cannot be excluded. The staining intensity, in this case, seemed much less than that of median-saccule and lysosomal membranes. Further technical progress allowing simultaneous detection of both membrane antigens and acid phosphatase activity would be needed to clarify this point. All these immunocytochemical observations suggest that these membrane do-

main possess a major antigen (mol wt 135,000) in common. Further studies with monoclonal antibodies will permit more refined identification of these components. In addition and as already observed in NRK cells (10), discrete areas of the plasma membrane were also labeled with the A-Golgi antibodies and positive vesicles were sometimes seen fusing with the plasma membrane. This suggests that some of the labeled vesicles may play a role in the transport of material from the Golgi zone to the cell surface.

In contrast, the A-Golgi antibodies did not label the secretory granule membrane. One cannot exclude the possibility that the fixative did not equally permeate all intracellular membranes. However, the presence of discrete positive membrane staining at the level of a very few segregating granules speaks in favor of a change in membrane composition at an early step in the packaging of secretory granules.

When applied to GH₃ cells, the two immunological probes

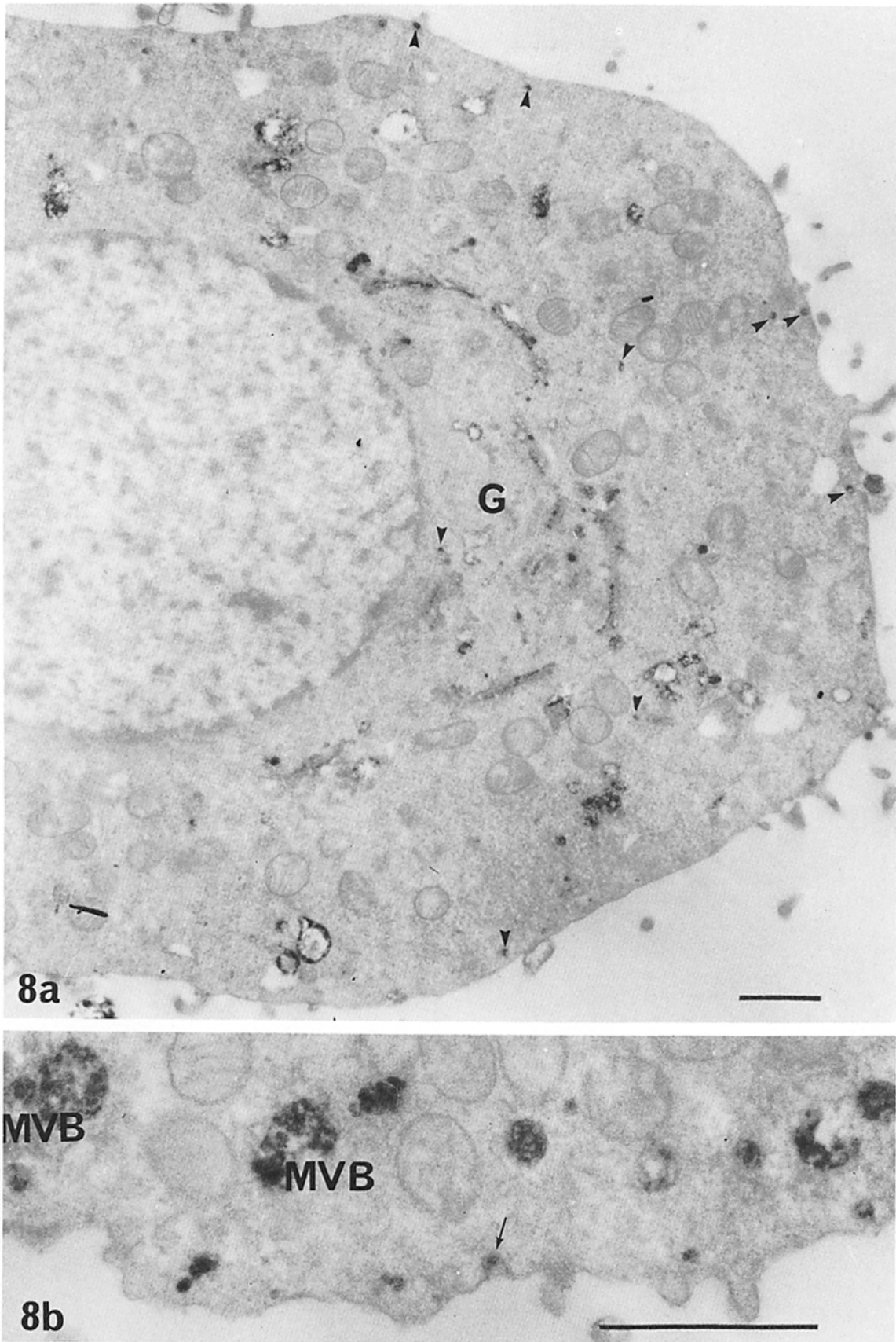
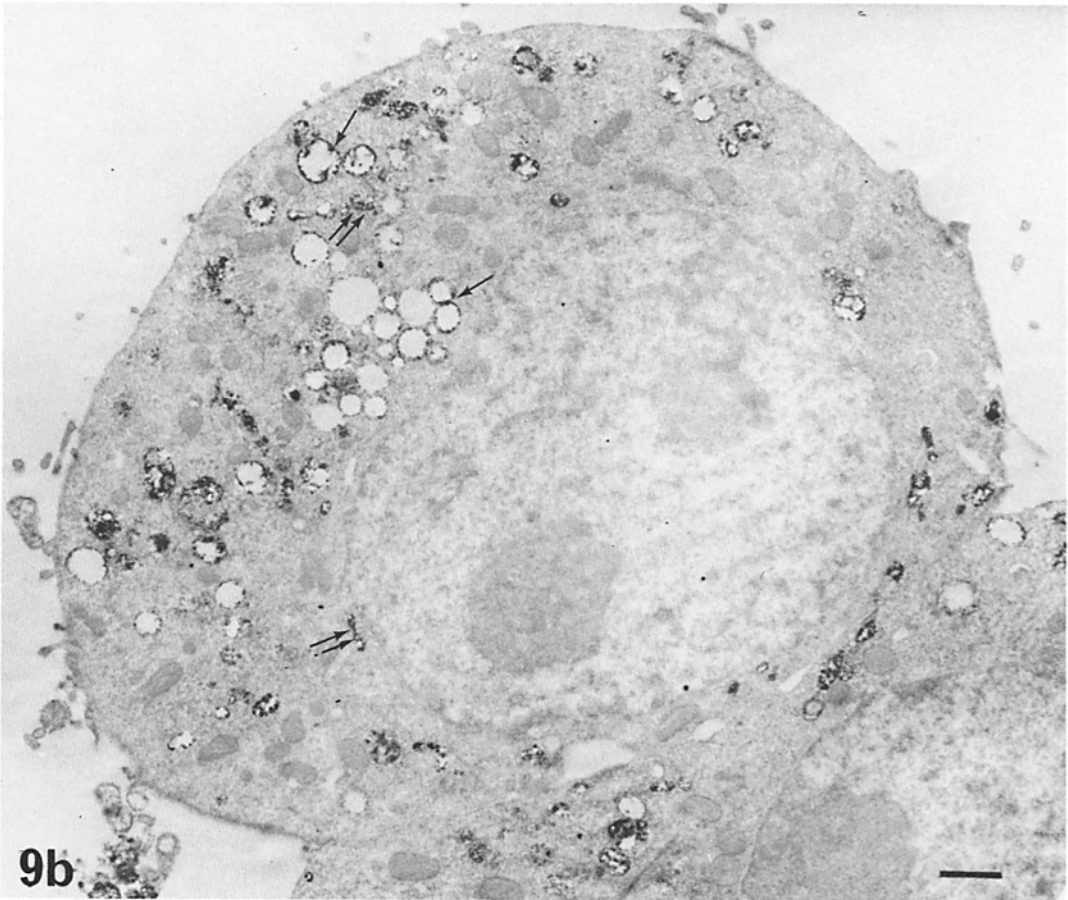
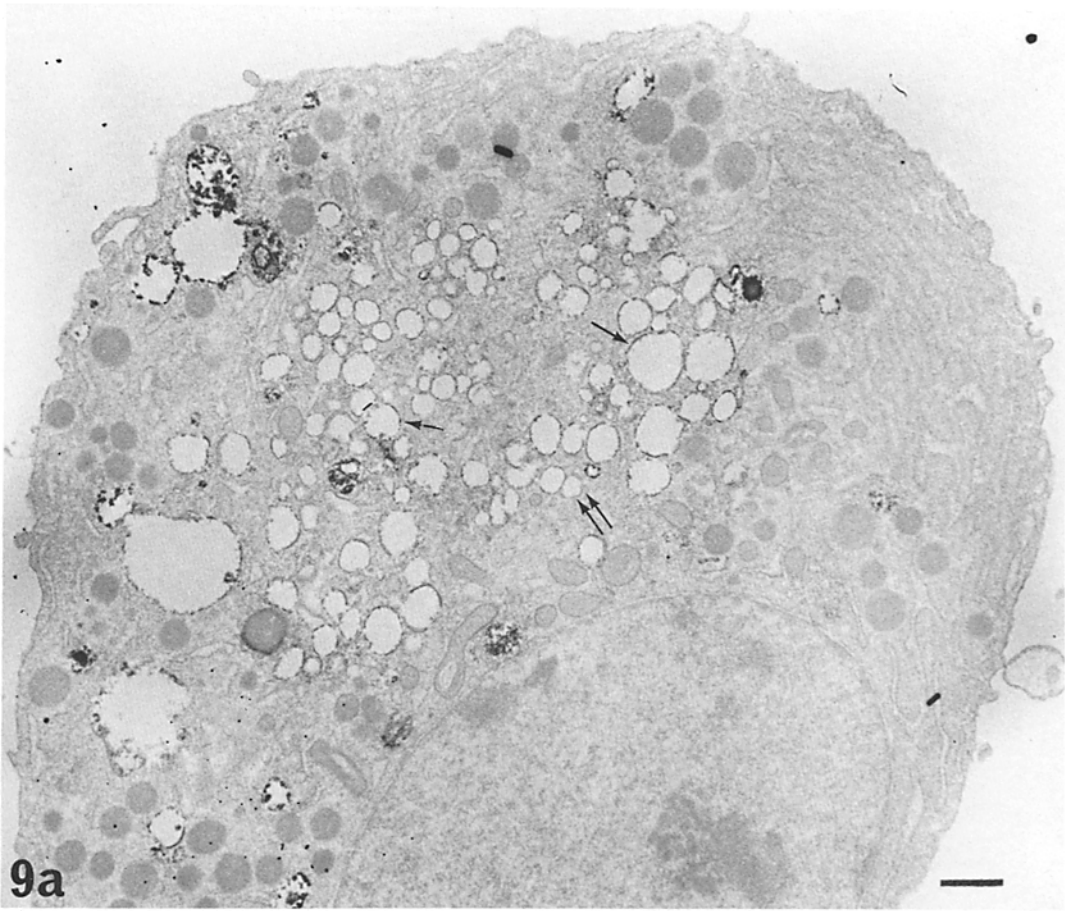


FIGURE 8 GH₃ cells exposed to TRH for 1 h immunochemically stained with anti-Golgi antibodies as in Fig. 7. (a) TRH treatment induced an extension of the Golgi zone (G) and an increased number of immunoreactive small vesicles (arrowheads) dispersed in the cytoplasm and beneath the plasma membrane. Bar, 1 μ m. \times 13,500. (b) Same material at a higher magnification. Some immunoreactive small vesicles (arrows) were observed fusing with the plasma membrane. The membrane of multivesicular bodies (MVB) was labeled as well as their inner vesicles. Bar, 1 μ m. \times 36,000.



gave results consistent with those obtained by conventional electron microscopy (8) and by immunocytochemical visualization of intracisternal PRL (24). Moreover, there is a good homology for the distribution of membrane components in both normal and tumor-derived prolactin cells.

An important aspect of the usefulness of the immunological probes concerns their application to PRL cells undergoing alteration of their secretory activity. Under acute stimulation of PRL release by TRH, the A-Golgi antibodies revealed in both normal and tumor-derived PRL cells an extension of the Golgi zone with an increase in number of small vesicles dispersed in the cytoplasm and beneath the plasma membrane. This is in favor of an increased membrane flow, which is consistent with previous studies using other approaches (8, 21). Morphometric studies would be needed to ascertain this conclusion. An increased number of PRL-loaded small vesicles has also been previously observed in TRH-treated cells (24). Whether the A-Golgi-labeled vesicles are also loaded with PRL remains to be demonstrated.

The origin of monensin-induced vacuoles with respect to Golgi subcompartments remains a matter of speculation. This drug, which is known to perturb Golgi traffic (18), also affects PRL secretion in GH₃ cells (25). Under these conditions the membrane compartment stained by A-RER antibodies was not affected, whereas A-Golgi antibodies labeled the membranes of the dilated vacuoles that accumulate in the perinuclear area as well as elongated cisternae (Fig. 9b). After PRL immunostaining, most of the monensin-induced vacuoles were labeled. On the contrary, acid phosphatase activity was restricted to lysosomes, dense bodies, and to some discontinuous elongated cisternae undilated by monensin (25). In conclusion, the A-Golgi antibodies labeled both a monensin-sensitive and less-sensitive smooth membrane compartments.

In conclusion, the antibodies directed against membrane components of the RER and of the Golgi zone appear to be useful tools in the analysis of membrane traffic in connection with the flow of secretory products in PRL cells. They provide new direct evidence for a sorting of membrane components in the Golgi zone: the 135-kdalton protein is directed to lysosomes and plasma membrane but not secretory granule membranes. It is known that the sorting of secretory material (PRL) and lysosomal hydrolases occurs at the same location (4, 6, 24). The present work suggests that more refined immunological tools will permit further analysis of the role of the Golgi zone in the secretory pathway.

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FIGURE 9 Monensin-treated prolactin cells immunochemically stained with anti-Golgi antibodies. (a) In a normal prolactin cell exposed to monensin for 1 h the anti-Golgi antibodies labeled the membrane of many large and dilated vacuoles (arrows) induced by the treatment. Some of these vacuoles were unlabeled (double arrows). Bar, 1 μ m. \times 8,000. (b) In a GH₃ cell exposed to monensin for 1 h the anti-Golgi antibodies labeled the membrane of many large and dilated vacuoles (arrows) as well as some undilated elongated structures (double arrows) in the disorganized Golgi region. Bar, 1 μ m. \times 8,000.