IMMUNOGLOBULIN SYNTHESIS AND SECRETION

II. Radioautographic Studies of Sites of Addition of Carbohydrate Moieties and Intracellular Transport

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ABSTRACT

The subcellular sites of synthesis and route of intracellular transfer of immunoglobulin G (IgG) have been investigated by electron microscope radioautography with precursors used for the polypeptide chain (leucine-^{3}H) and for the carbohydrate moieties (galactose-^{3}H and glucosamine-^{3}H). For this purpose, plasma cells from a mouse myeloma tumor were labeled with appropriate precursors and the distribution of radioautographic grains was determined at the end of the labeling period and after varying times of incubation in unlabeled medium. The results indicated that the polypeptide backbone is synthesized in a region of the cell occupied by the rough endoplasmic reticulum (RER) and is transported from there to the region of the Golgi complex. Galactose is incorporated in IgG primarily at the level of the Golgi complex, whereas the incorporation of glucosamine appears to take place both in the RER and in the Golgi complex. From the Golgi complex, the completed IgG molecules reach the plasma membrane and are discharged extracellularly. The latter route of transport and the mechanism of discharge are not understood but may be mediated via smooth-surfaced vesicles.

INTRODUCTION

In the biochemical studies reported in the accompanying paper, (1) evidence was obtained that galactose is added onto completed polypeptide chains of the immunoglobulin molecule (IgG) late in its intracellular life, whereas glucosamine, a "bridge" sugar, appears to be incorporated into both nascent and completed polypeptide chains of IgG.

The present study was designed to elucidate the intracellular route of transport of the polypeptide portion of IgG and to define the sites of incorporation of glucosamine and galactose into the carbohydrate portion of IgG. For this purpose, cells from the mouse plasma cell tumor LPC_1 were labeled in vitro with leucine-^{3}H, galactose-^{3}H, or glucosamine-^{3}H and were examined by electron microscopic radioautography at the end of the labeling period and after various times of incubation in unlabeled medium.[1]

[1] A preliminary report of these results has been published (2).

MATERIALS AND METHODS

Preparation of Cell Suspensions

LPC_1, kindly provided by Dr. John Fahey, is a transplantable plasma cell tumor producing γG_2B which is carried in Balb/c mice (3). 10–17 day old subcutaneous tumors were removed and placed in Eagle's medium (Grand Island Biological Co., Grand Island, N. Y.) without glucose and with 1/100 × amino acid concentration unless otherwise indicated. Glucose was omitted in order to enhance incorporation of labeled sugars. Cells were teased from the tumors with a scalpel, filtered through a stainless steel screen, washed three times by centrifugation in the same medium at 200 *g* for 6 min, and finally resuspended at a concentration of 2×10^7 cells/ml. The suspension was preincubated at 37°C for 30 min in the above medium before labeling. Cells were routinely examined by phase-contrast microscopy after exposure to trypan blue to assess uniformity and viability of the cell populations used for each experiment. Approximately 30% of the cells derived from the tumor were alive, and this percentage was maintained during the 1–2 hr of experimentat incubation.

Labeling Procedure

Cells were labeled with either leucine-^{3}H (L-leucine-4,5-^{3}H, 45 Ci/mmole), galactose-^{3}H (D-galactose-1-^{3}H, 6.9 Ci/mmole), or glucosamine-^{3}H (D-glucosamine-6-^{3}H), 1.1 Ci/mmole) obtained from the New England Nuclear Corp., Boston, Mass. Final concentration of labels per ml of media was 200 μCi for leucine, 500 μCi for galactose, and 800 μCi for glucosamine. After labeling with leucine-^{3}H, further incorporation of isotope was terminated by adding a 500-fold excess of leucine-^{1}H followed by 3×10^{-4} M cycloheximide 2 min later (chase incubation medium). The leucine chase was effective within <30 sec. In contrast, attempts to chase labeled sugars with high concentrations of unlabeled sugars were only partially effective. Consequently, following exposure to labeled sugars, the cells were simply washed free of label and resuspended in Eagle's medium containing glucose.

To determine incorporation of labeled leucine or sugars into protein, aliquots of the unwashed cell suspensions and their incubation media (termed secretion, henceforth) were precipitated with 5% trichloroacetic acid (TCA; final concentration) and washed three times with cold 5% TCA. In addition, acrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate was performed on the IgG precipitated by specific antibody from cytoplasmic extracts and secretions (1). The gel was divided according to the method of Maizel (4). Radioactivity was measured by counting in a Beckman liquid-scintillation counter in Bray's phosphor (5).

Preparation of Cells for Radioautography

Cells were fixed in cold 1% OsO_4 in 0.1 M phosphate buffer, pH 7.4, for 1 hr following a single wash with unlabeled incubation medium. This washing-fixation schedule appeared to remove effectively any unincorporated, soluble label which could be artefactually fixed into the tissue. Support for this statement is that incubation of cells pretreated with puromycin completely prevented appearance of grains after incubation with leucine and markedly depressed (approximately 90%) the appearence of grains after incubation with glucosamine and subsequent fixation. In addition, mixing experiments showed that exposure of unlabeled cells to incubation medium containing labeled IgG did not result in the artifactual adsorption and subsequent fixation of macromolecules onto the cell surface. The fixed cells were pelleted by centrifugation, dehydrated in alcohol, and embedded in Epon (6). Thin sections of medium-yellow interference color were mounted on Formvar-coated grids backed by a thin layer of carbon.

Electron microscope radioautography was performed according to the method of Caro and van Tubergen (7). Radioautographic exposure times were ~3 wk for leucine-^{3}H-labeled samples and ~5 wk for both galactose- and glucosamine-labeled material. Microdol-X (Eastman Kodak Co., Rochester, N. Y.) was employed as the developer, followed by Kodak acid fixer. Prior to staining with uranyl acetate and lead citrate (8), the emulsion was removed by briefly soaking the grids in 0.1 N NaOH.

Only those cells whose fine structure appeared adequately preserved were used for radioautographic grain counting. Cell debris and cells whose nuclei appeared vacuolated or pyknotic, or whose cytoplasmic organelles were grossly damaged were disregarded. During the 2-hr incubation period used in these experiments, the majority of the cells appeared morphologically intact.

The per cent distribution of radioautographic grains was determined over the following four general areas of the cell. (*a*) Nucleus. (*b*) Plasma membrane: grains falling over a band 0.2 μ wide on either side of the plasma membrane were assumed to be associated with the plasma membrane. (*c*) Golgi region: this region of the cell is usually located, in plasmacytes, in the nuclear indentation and is identified by its complement of smooth-surfaced cisternae and vesicles. (*d*) The remaining cytoplasm: this includes the rough endoplasmic reticulum (RER) and mitochondria, as well as the surrounding cytoplasmic matrix and its population of free polysomes (9, 10). It also includes occasional clusters of viral particles usually found in such cells (11).

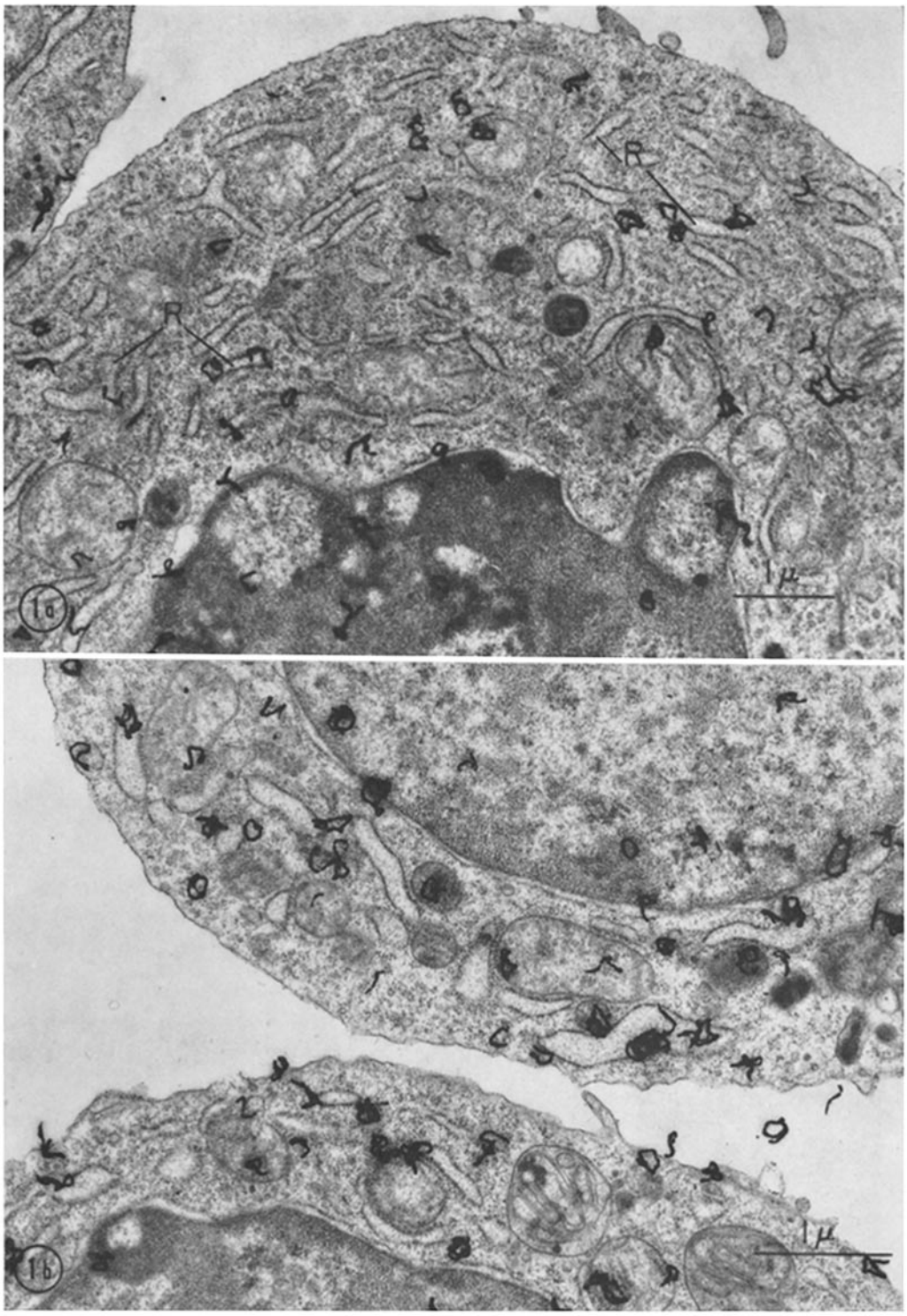

FIGURE 1 *a–b* LPC$_1$ plasma cell after pulse labeling with leucine-^{3}H for 5 min. Note that the silver grains mainly mark the cytoplasm and appear to be associated with elements of the rough endoplasmic reticulum (*R*). This is particularly well seen in Fig. 1 *b* where silver grains are frequently associated with dilated RER cisternae. At this time, the Golgi complex is mainly unlabeled. Fig. 1 *a*, × 14,500. Fig. 1 *b*, × 20,000.

RESULTS

Characterization of Biosynthetic Activities

The studies reported in the companion paper (1) established that glucosamine-^{3}H and galactose-^{3}H become covalently bound to IgG; the attached label is recoverable as the precursor sugar (or in the case of glucosamine, as *N*-acetyl glucosamine), and virtually all the label that becomes covalently associated with macromolecules is attached to IgG.

The proportion of leucine-^{3}H incorporated into macromolecules that becomes associated with IgG was determined by labeling LPC_1 cells for 30 min and measuring both total acid-precipitable radioactivity and radioactivity which is precipitated by antiserum specific to mouse IgG. The results indicated that ~50% of the leucine-^{3}H incorporated into protein represents IgG synthesized during exposure to the label. On the other hand, all of the leucine-^{3}H–labeled material secreted by the cells is IgG.

As noted above, the fixation schedule effectively removes unincorporated soluble label from the cells. This fact, plus the biosynthetic studies summarized above, indicate that the distribution of radioautographic grains following labeling with sugar precursors will mark almost exclusively the position of newly formed IgG molecules. With leucine-^{3}H as precursor, only ~50% of the label represents IgG, the remainder presumably being incorporated into sedentary nonexportable proteins. Since IgG is the only molecule transported and secreted by these cells, however, changes in the pattern of labeling of cell components during chase incubation will reflect in large part the fate of IgG molecules labeled with leucine-^{3}H.

Finally, it should be noted that within a given experiment, the intracellular transport and secretory functions of all cells in the suspension appeared to operate at similar rates.

Incorporation of Leucine-^{3}H

At the end of a 5 min pulse, the majority (~57%) of the radioautographic grains were associated with the cytoplasm (Fig. 1 and Table I). Of these, approximately 65% were associated with cisternal elements of the RER. At this time, only a small percentage of the label (~3%) was associated with elements of the Golgi complex, the remainder being equally divided between the nucleus and a zone ~0.2 μ wide spanning the plasma membrane. During a subsequent 30 min chase incubation, the distribution of radioautographic grains was markedly changed: at this time ~27% of the label was associated with elements of the Golgi complex (Fig. 2) and the proportion remaining over the cytoplasm (~28%) was decreased by about one-half. Approximately 20% of the grains over the cytoplasm were associated with elements of the RER. There was no marked change in the label associated with the nucleus and plasma membrane.

Incorporation of Galactose-^{3}H and Glucosamine-^{3}H into Plasma Cells

With the sugar precursors employed in this study the total amount of isotope incorporated,

TABLE I

Incorporation of Labeled Precursors into Organelles of LPC_1 Plasma Cells as Observed by Electron Microscope Radioautography

Isotope	Duration of pulse chase		Total grains counted	Distribution of radioautographic grains			
				Cytoplasm	Golgi	Plasma membrane	Nucleus
^3H	*min*			%	%	%	%
Leucine	5	0	1656	57	3	12	14
	5	30	1326	28	27	15	13
Galactose	60	0	483	16	51	10	13
	60	90	499	23	28	38	9
Glucosamine	60	0	872	41	12	5	36
	60	90	296	25	36	11	21

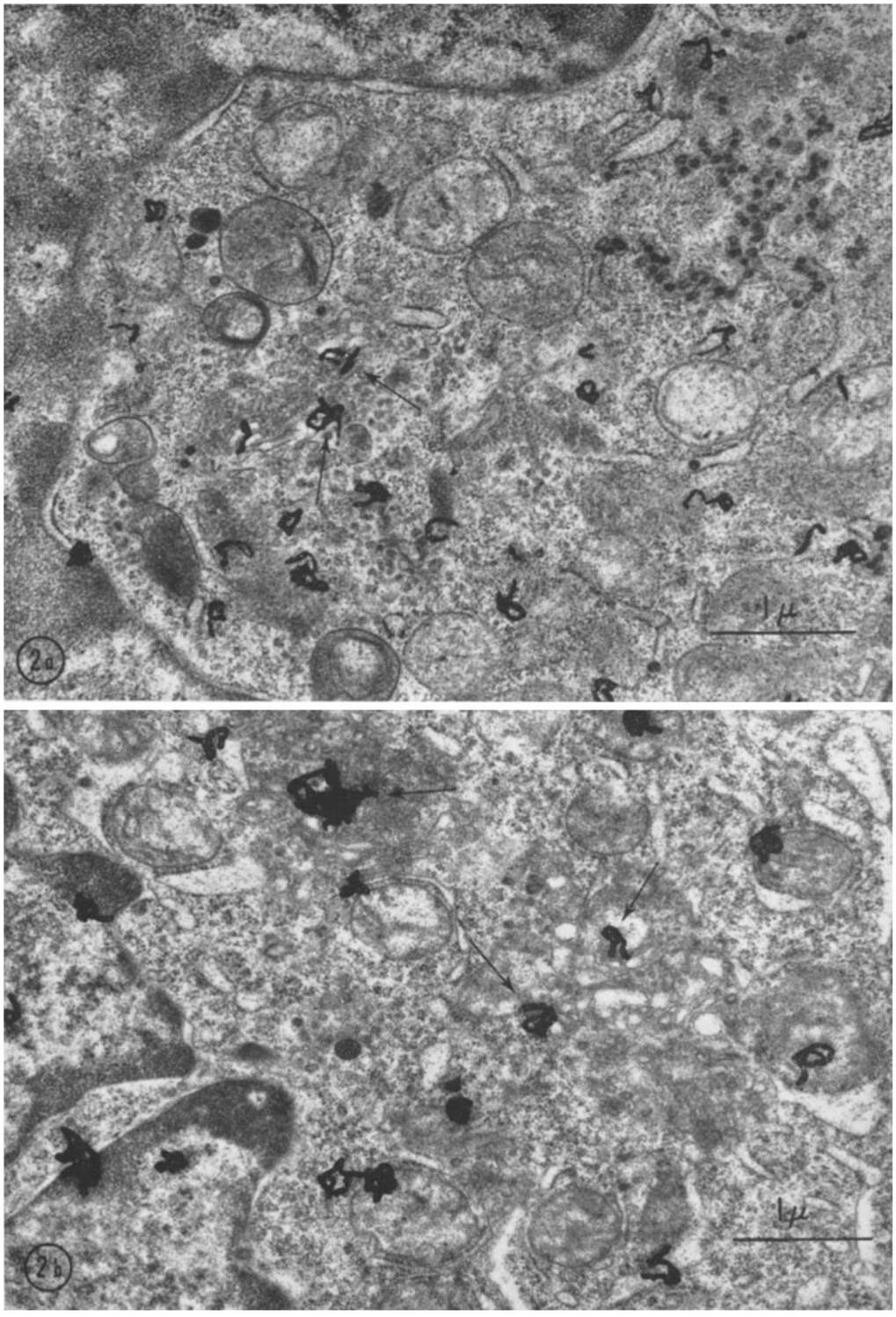

FIGURE 2 *a–b* LPC_1 cells pulse labeled with leucine-^3H for 5 min and incubated in chase medium for 30 min. At this time, the radioautographic label appears over elements of the Golgi complex (arrows). Fig. 2 *a–b*, × 20,000.

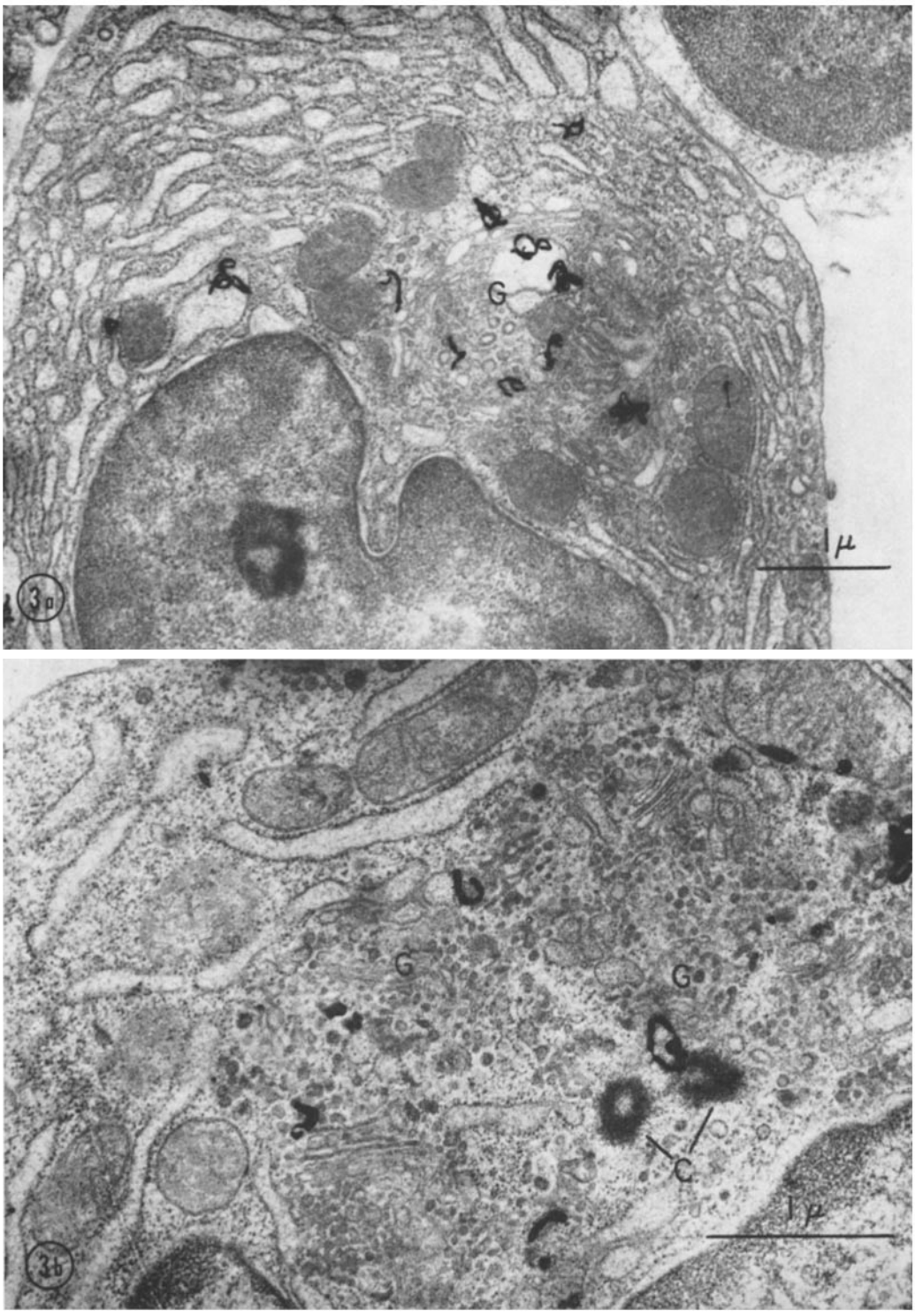

FIGURE 3 *a* LPC$_1$ cell labeled with galactose-^{3}H for 60 min. Radioautographic grains are primarily located over the Golgi complex (*G*). × 20,000.

FIGURE 3 *b* As in Fig. 3 *a*, but exposed to galactose-^{3}H for 15 min. *C*, centrioles. × 28,000.

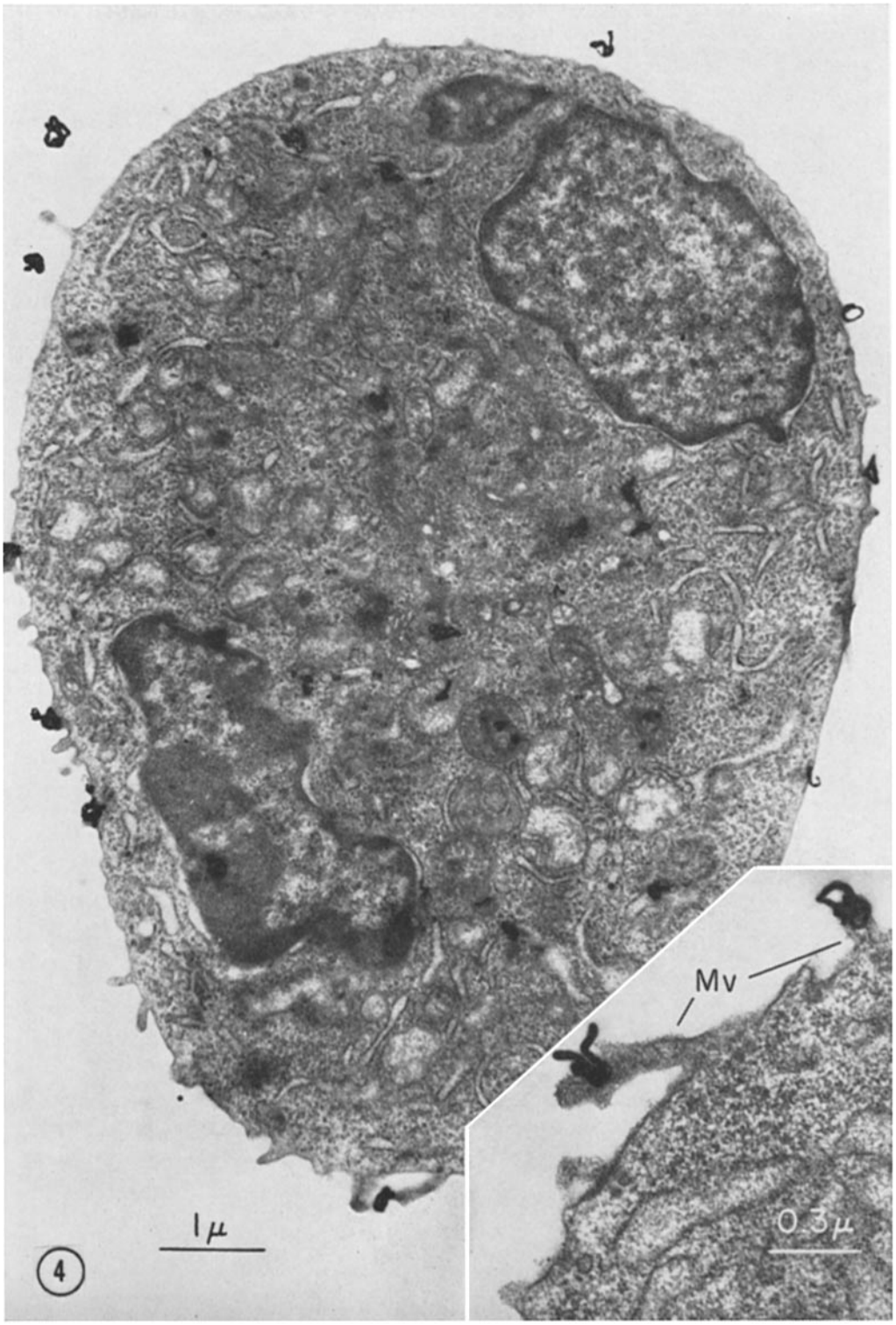

FIGURE 4 LPC_1 cells labeled with galactose-^3H for 30 min and incubated for 90 min in unlabeled medium. This figure shows the accumulation of label over the plasma membrane and in association with microvillar projections (*Mv*) (see *insert*). Some label is still associated with elements of the Golgi complex at this time. Fig. 4, × 16,000. *Insert*, × 45,000.

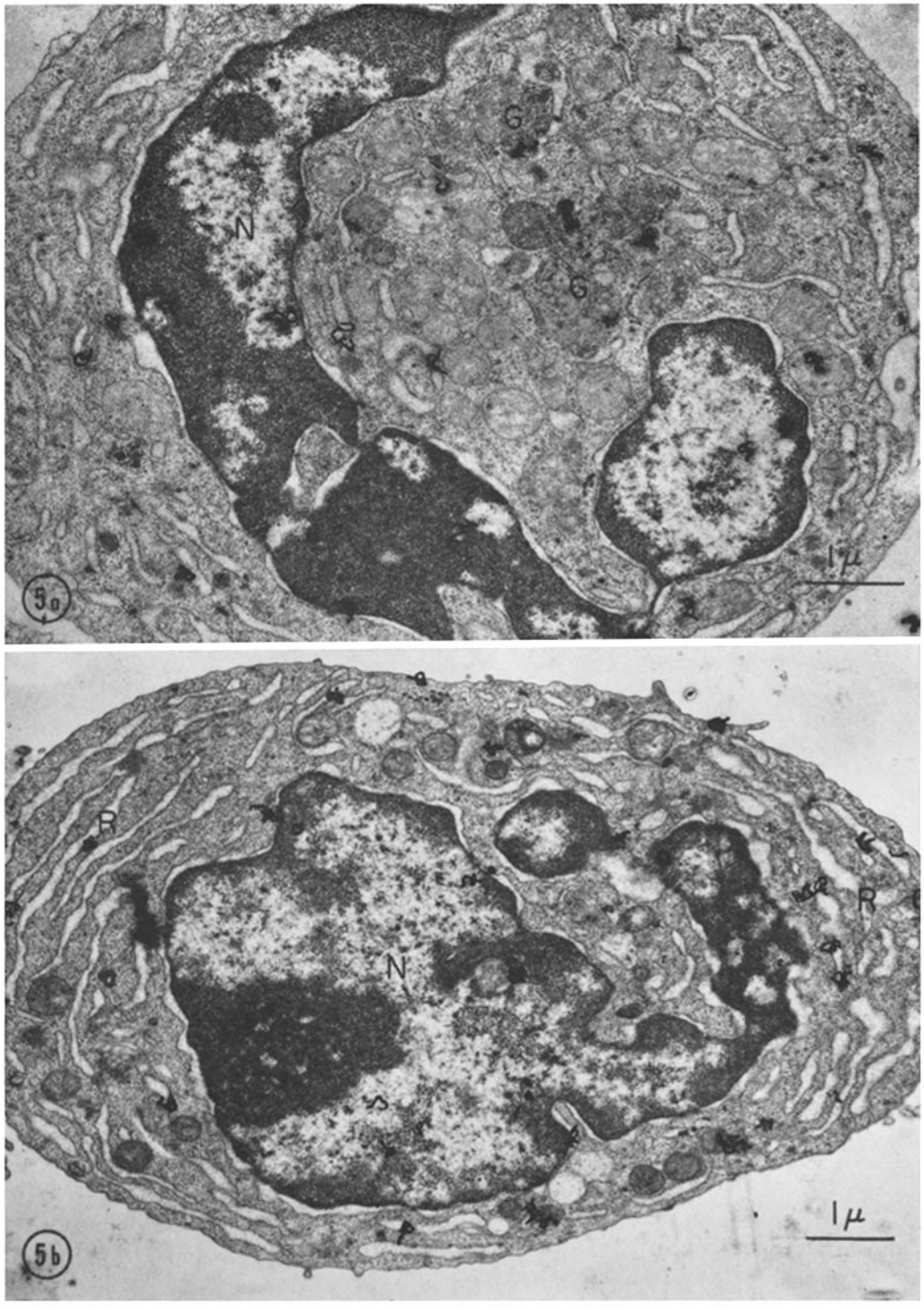

FIGURE 5 *a–b* LPC_1 cells labeled with glucosamine-^3H for 60 min. Grains are located mainly over the RER (*R*) with some label over the Golgi complex (*G*) and the nucleus (*N*). Fig. 5 *a*, × 14,500. Fig. 5 *b*, × 13,000.

compared to leucine-^{3}H, is substantially smaller. Hence, to obtain an adequate grain count within reasonable exposure times (3–5 wk), we were obliged to extend the labeling time with sugar precursors to 60 min. Parallel experiments with both light and electron microscope radioautography were used to evaluate the distribution of each precursor after exposure to label for 5–30 min.

Following 60 min of labeling with galactose-^{3}H, the majority of the radioautographic label (51%) was associated with elements of the Golgi complex, the remainder of the label being distributed evenly over the cytoplasm, nuclei, and plasma membrane (Table I). A qualitatively similar distribution was seen as early as 5 or 15 min after labeling with galactose-^{3}H (Fig. 3). After an additional 90 min incubation of the cells in medium lacking the isotope, the proportion of label over the Golgi complex had decreased, while that associated with the plasma membrane had increased markedly (~38%) (Fig. 4). A modest increase in the percentage of grains over the cytoplasm was noted at this time.

Control experiments showed that the relatively long incubation times employed had not significantly depleted galactose-^{3}H from the incubation medium. Consequently, the small proportion of grains over the cytoplasm, particularly the RER, was not caused by an inadvertent chase due to depletion of galactose.

These data thus show that the initial site of incorporation of galactose-^{3}H into plasma cells is the elements of the Golgi complex and indicate that the RER is probably not involved in addition of galactose to IgG.

Incorporation of Glucosamine-^{3}H

After 60 min of labeling, grains were localized to the cytoplasm (41%), elements of the Golgi complex (12%), and the nucleus (36%) (Fig. 5), especially the nucleolus (Table I).

This pattern of labeling is thus intermediate between those observed with leucine-^{3}H and galactose-^{3}H. In view of the biochemical data presented in the previous paper, we interpret this pattern to mean that there are likely two sites of incorporation of glucosamine-^{3}H into IgG: a proximal site associated with polyribosomes or at least located at the level of the RER cisternae, and a distal one possibly located at the level of the Golgi complex. This conclusion would be consistent with the fact that the qualitative distribution of radioautographic grains after 15 min of incubation is similar to that at 60 min of labeling and is compatible with the observation that after 90 min of incubation in unlabeled medium the proportion of grains over the Golgi region increases by ~threefold.

DISCUSSION

Several lines of evidence indicate that electron microscope radioautography performed on cells from mouse plasma cell tumors should enable us to elucidate the sites of biosynthesis and route(s) of secretion of IgG: (*a*) the cell population, which presumably represents a single clone (12), appears homogeneous in its biosynthetic capacities; (*b*) the only protein secreted by these cells is IgG; (*c*) virtually all of the galactose-^{3}H and glucosamine-^{3}H incorporated into the cytoplasm of the cells is covalently bound to IgG (glucosamine in the *N*-acetyl form). Although only ~50% of the leucine-^{3}H incorporation is into IgG, the fact that IgG is the only protein secreted by these cells enables us to determine the route(s) of intracellular transport of this molecule against a background of presumed sedentary, nonexportable proteins. These biosynthetic studies, therefore, aid in the interpretation of the labeling patterns seen by electron microscope radioautography.

Studies with leucine-^{3}H have enabled us to determine at least some of the steps involved in the intracellular transport of IgG through the cell. The distribution of radioautographic grains at the end of 5 min of pulse labeling indicates that the peptide portion of IgG is synthesized in the cytoplasm in association with elements of the RER. This finding would be predicted by previous work on this system (13, 14, 9, 10) and by studies in other cell types producing proteins for export (15, 16). Following 30 min of chase incubation, the proportion of label remaining in the cytoplasm decreased and was accompanied by a corresponding increase (to ~27%) in the proportion of label associated with elements of the Golgi complex. Similar results were reported by Clark, using lymph node cells from immunized rabbits (17). Although the limitation of resolution of the radioautographic technique does not allow us to determine precisely the localization of labeled IgG in the Golgi complex, cell fractionation studies on other exocrine cells indicate that in this location the secretory proteins are in transit through the

cell within the content of the vesicles, vacuoles, and cisternae which comprise the Golgi complex (18).

In contrast to the situation with leucine-^{3}H as precursor, galactose-^{3}H was initially incorporated at the level of the Golgi complex, with only a small amount of label being associated with the cytoplasm. This localization is consistent with the biochemical studies reported in the previous paper and supports the generalizations reached by others that the Golgi complex plays a central role in the synthesis of the carbohydrate moieties of exportable glycoproteins (19, 20).

The pattern of labeling by glucosamine-^{3}H is in between those seen with leucine-^{3}H and galactose-^{3}H. After labeling for 60 min, a substantial percentage of the radioautographic grains is associated with elements of the RER in the cytoplasm and a small but significant proportion appears to be located over the Golgi complex. This latter localization is consistent with biochemical studies (1) which show, by means of puromycin pretreatment, that glucosamine is incorporated into completed polypeptide chains and suggest that it is also incorporated into nascent chains as well. Hence, we conclude that glucosamine (as *N*-acetyl glucosamine) is incorporated into IgG, most likely at the level of polyribosomes attached to membranes of the RER, as well as at a more distal site possibly located at the Golgi complex. Proof for the latter site of addition would necessitate demonstrating glucosamine label in the Golgi complex in cells pretreated with puromycin in which IgG has been transported out of the RER before labeling. During 90 min of incubation without the precursor, the amount of label located over the cytoplasm decreases while that over the Golgi complex increases. Again, as in the case of IgG molecules labeled with leucine-^{3}H, this finding would indicate that the polypeptide chains glycosylated in the RER eventually are transported to the Golgi complex. The significance of the large amount of label incorporated into the nucleus (primarily the nucleolus) is unknown.

The sequence of events involved in the formation of the carbohydrate portion of IgG reported here differs from that advanced by Swenson and Kern (14, 21). While these authors showed that labeled amino acids were incorporated into IgG present most likely in "microsomal fractions" from lymph node cells, their data suggested that glucosamine and galactose are both incorporated into IgG free in the cell sap. This implies that the completed polypeptides, having been segregated in the cisternae of the RER (22), must again cross the RER membrane to enter the cell sap prior to glycosylation. Such a mechanism is inconsistent with available evidence on the fate of proteins destined for export in other cell types (18, 23, 24), which shows that the accumulation of peptide chains in the RER space is an apparently irreversible step. In addition, cytochemical studies by others (9, 10, 25) on plasma cells have clearly indicated that immunoglobulins are apparently undetectable free in the cell sap, but are localized to both the RER cisternae and elements of the Golgi complex.

Our studies show that the Golgi complex is a way-station in the intracellular transport of IgG. From here, it reaches the incubation medium (i.e., extracellular space). According to the studies of Helmreich et al. (26, 27) immunoglobulins are secreted from plasma cells without cell disruption. In contrast to the situation in many exocrine and endocrine cells, where secretory products are concentrated and temporarily stored in secretory granules, cells such as the plasmacyte appear to transport and discharge continuously without concentration and storage of their product (28, 29, 26). At present, the mechanism of discharge is not understood, though two alternatives can be considered. First, the IgG molecules, upon completion of glycosylation in elements of the Golgi complex, enter the cell sap from where they are discharged in soluble form across the plasma membrane. As mentioned above, however, cytochemical studies do not support this route. Second, Golgi-derived vesicles filled with IgG may migrate to the cell surface, fuse with the plasma membrane, and discharge their contents by "reversed pinocytosis"—i.e., by a mechanism similar to the discharge of secretory products from storage granules (30). We favor this latter mechanism although morphological evidence to support this route is lacking in plasma cells and unfortunately the limitations of the radioautographic technique will not enable us to determine if this is the case. A final solution to this problem will depend on cell fractionation techniques.

Finally, we should point out that during longer times of incubation in unlabeled media, silver grains frequently marked the plasma membrane and its microvilli. It is possible that IgG molecules, following discharge by the mechanism proposed above, are temporarily attached to the plasma membrane prior to their release to the extracellular space.

This work was done under the sponsorship of the Commission on Immunization of the Armed Forces Epidemiological Board, and was supported in part by the United States Army Medical Research and Development Command, Department of the Army, under Research contract DADA 17-69-C 9177, and in part by United States Public Health Service Grants AI-08034, HE-05648, AM-10928, and by National Science Foundation Grant GB-7473-X.

Received for publication 17 November 1969, and in revised form 4 February 1970.

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