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Immunoglobulin Synthesis and Secretion, IV. Sites of Incorporation of Sugars as Determined by Subcellular Fractionation*

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Abstract. P_3K cells were uniformly labeled with [^{14}C]leucine and also for 1 hr with [^{3}H]leucine, [^{3}H]galactose, or [^{3}H]glucosamine. Subcellular fractionation of labeled cells was performed and the amounts of radioactive immunoglobulin G in each fraction were determined. The results indicate that amino acids are incorporated in the rough endoplasmic reticulum, galactose primarily in the smooth endoplasmic reticulum, and glucosamine at both sites. A small portion of radioactivity was found in the microsomal supernatant regardless of the radioisotope employed.

Previous studies with myeloma cells LPC_1 have shown that sugars are incorporated into immunoglobulin G (IgG) at two sites: the bridge sugar, *N*-acetylglucosamine, is incorporated into nascent chains on polyribosomes[1] after which the IgG molecule is transported through the cisternae of the rough endoplasmic reticulum to the Golgi complex where galactose is added.[2,3] Some glucosamine residues are also incorporated on completed chains, possibly in the Golgi complex. These findings show that incorporation of sugars occurs primarily if not exclusively within organelles.[4]

In contrast, as a result of fractionation studies on rabbit lymph nodes, Swenson and Kern have postulated that sugars (including glucosamine and galactose) are incorporated into IgG in the cell cytoplasm.[5] They found that virtually all IgG labeled with radioactive sugars appeared in the microsomal supernatant, whereas IgG labeled with leucine was present almost exclusively in microsomes.

We have attempted to resolve this fundamental difference in concept by performing subcellular fractionations similar to those of Swenson and Kern on an established myeloma cell line that forms IgG. The results are consistent with those we have previously reported. Further, in contrast to Swenson and Kern, we have found most of the radioactive IgG to be associated with the microsomes, regardless of precursor employed.

Materials and Methods. Labeling of cells: P_3K, an established line of BALB/C murine myeloma cells which secretes IgG, was obtained from Dr. Kengo Horibata at the Salk Institute. The cells were grown in suspension culture in Dulbecco's medium (Grand Island Biological Laboratories, N.Y.) with 10% horse serum and had a generation time of 16–20 hr. They were collected by centrifugation, 500 × *g* for 10 min, and were washed twice in Eagle's medium (Grand Island Biological Laboratories) lacking

leucine. For double labeling, 10 ml of washed cells (10^7/ml) was resuspended in the same medium, exposed to 1.5–3 μCi/ml of [^{14}C]leucine (240 mCi/mM) (New England Nuclear) for 60 min at 37°C, and washed twice in Eagle's media lacking glucose and leucine but containing [^{14}C]leucine at the above concentration. Equal aliquots of 3.3 ml, 10^7/ml were labeled with either 30 μCi/ml ^{3}H-D-leucine (58 Ci/mM), 75 μCi/ml ^{3}H-D-glucosamine (1 Ci/mM), or 75 μCi/ml ^{3}H-L-galactose (7 Ci/mM) (New England Nuclear). Cell viability as determined by exclusion of trypan blue was greater than 90% throughout the labeling period. Labeling was terminated by dilution of cells into medium at 4°C. Cells were washed once in Eagle's medium.

For "chase" experiments, Eagle's medium lacking valine but containing 25 μCi/ml of ^{3}H-L-valine (2 Ci/mM) was used. Cycloheximide (Upjohn) was added to a concentration of 100 μg/ml of suspension.

Subcellular fractionation: 3.0 ml of labeled cells were resuspended in 2.5 ml of 0.88 M sucrose in distilled water (initial pH 5.5). The flow diagram of the fractionation procedure is presented in Figure 1. Following conventional separation of the major organelles, microsomes were subfractionated on a discontinuous sucrose gradient in the SW-56 rotor as described by Tartakoff.[6] An excellent separation of rough from smooth endoplasmic reticulum was obtained and the purity was confirmed by electron microscopic examination. Fractions were removed from the top of the tube with a capillary pipet.

A 0.3-ml aliquot of the initial cell suspension was treated with Nonidet P40 (NP40) (Shell Company) at a concentration of 0.5% for 10 min at 4°C. Nuclei were pelleted by centrifugation at 800 × g for 10 min; the supernatant is referred to as the cell lysate. In some experiments, aliquots of the dissolved specific precipitates were treated with sodium dodecyl sulfate and electrophoresed in acrylamide gel in the presence of this buffer.[2]

Chromatography of sugars: Hexoses from labeled heavy chains[7] were separated from an acid hydrolysate of the glycoprotein (100°C, 4 N HCl, 6 hr) on Dowex 50 (H+ form) according to the method of Boas *et al.*[8] and identified by thin-layer chromatography (MN 300, cellulose normal; Analtech Inc.) with BuOH-pyridine-H_2O (6:4:3 v/v) as solvent. Sugar standards (Mann Research) were used and simple as well as cochromatography was performed in all cases. Visualization was by Partridge's reagent.[9]

Scintillation counting: Specific precipitates dissolved in acid or effluents from columns were added to Biosolve-3 (Beckman) and then to toluene-PPO.[10] Fractions from acrylamide gel electrophoresis or the scraped-off cellulose from thin-layer plates were added to Beckman cocktail D.[11] Determinations of radioactivity were done to the ±5% error in a Beckman LS-250 liquid scintillation counter.

Results. Chromatographic analyses of radioactive sugars incorporated into IgG: In order to interpret experiments in which [^{3}H]glucosamine and [^{3}H]-galactose were used as precursors, it was necessary to show that these sugars were each incorporated into IgG as

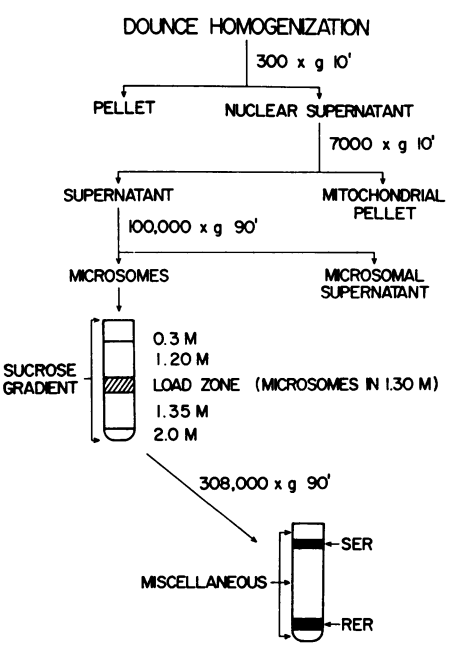

FIG. 1.

such. For this purpose, P_3K cells were incubated with each isotope for 60 min and the intracellular IgG was precipitated with specific antibody. Their final identification was as described in *Materials and Methods.* Isolation of the sugars in the heavy chain was performed as described previously.[2]

The studies indicate that galactose is incorporated as such and that glucosamine is incorporated as *N*-acetylglucosamine though a small amount can be detected as glucosamine. Since deacetylation of glucosamine can occur *in vitro*, it is presumed that glucosamine incorporation is entirely in its *N*-acetyl form. The results are in complete agreement with a similar chromatographic analysis of the incorporation of these sugars into IgG produced by another myeloma tumor, LPC_1.[2]

Fractionation of P_3K cells labeled with leucine or sugars: 10^8 P_3K cells were labeled with [^{14}C]leucine for 120 min. During the last 60 min either [^{3}H]leucine, [^{3}H]galactose, or [^{3}H]glucosamine was added to aliquots. The cells were fractionated and the amount of radioactive IgG in the various subcellular fractions was determined by specific precipitation. Aliquots of the precipitates were also analyzed by acrylamide gel electrophoresis and the results confirmed that precipitated radioactivity was virtually all IgG. Preliminary experiments had indicated that after 60–120 min [^{14}C]leucine had uniformly labeled intracellular IgG. The $^3H/^{14}C$ ratio of IgG in each subcellular fraction was independent of variations of recovery of the IgG and could thus provide a meaningful "relative specific activity."

Table 1 summarizes the results of a representative experiment and shows the counts per minute and the percentage distribution of IgG in each fraction.

With regard to [^{14}C]leucine, the percentage of distribution in subcellular fractions of IgG was generally similar in the three separate aliquots of cells, indicating that the methods involved in both fractionation and measurement are highly reproducible. Further evidence is

TABLE 1. *Distribution of radioactive IgG in subcellular fractions.*

Fraction	[^{3}H]leucine		[^{14}C]leucine		[^{3}H]galactose		[^{14}C]leucine		[^{3}H]glucosamine		[^{14}C]leucine	
	Dpm	Per cent*	Dpm	Per cent	Dpm	Per cent	Dpm	Per cent	Dpm	Per cent	Dpm	Per cent
Nuclear	133,543	13	37,110	15	16,395	16	45,853	17	1,970	10	33,263	12
Mitochondria	150,028	14	38,656	15	14,348	14	35,259	13	2,758	15	39,976	15
Microsomal supernatant	71,610	7	19,964	8	11,760	11	34,631	13	2,132	11	29,479	11
Microsomes:												
RER†	588,008	55	127,955	50	19,158	19	130,115	48	9,628	51	136,668	51
SER†	16,755	2	6,570	3	27,068	26	5,932	2	1,150	6	6,712	3
Misc.	97,395	9	23,289	9	14,385	14	20,065	7	1,290	7	20,083	8

* Per cent based on total recovery.
† RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum.

that total recoveries of the fractions in this and other experiments were usually equal to those predicted from determination of radioactive IgG in the whole cell lysate, i.e., an aliquot of the cells extracted with NP40. Since phase microscopy of the nuclear pellet indicated that cell breakage was virtually complete, the IgG in the nuclear as well as the mitochrondrial fraction probably arises from microsomes.

With regard to ^{3}H-isotopes, the distribution with [^{3}H]leucine after 60 min of labeling was similar to that of [^{14}C]leucine after 120 min of labeling, indicating that uniform labeling can be achieved in 60 min. Most of the radioactivity was in the microsomes rather than the microsomal supernatants regardless of the isotope used. For [^{3}H]leucine, the rough endoplasmic reticulum had 27 times more radioactivity than the smooth, whereas for galactose there was more radioactivity in the smooth endoplasmic reticulum. Glucosamine was intermediate to leucine and galactose. The percentage of IgG labeled by galactose in the nuclear and mitochondrial pellets was not significantly different from that of IgG labeled by leucine, suggesting that both smooth and rough endoplasmic reticula are present in these pellets.

Relative specific activities of IgG (^{3}H/^{14}C) in smooth and rough endoplasmic reticulum: These values were computed for the above experiment, two additional ones, and a fourth in which ^{3}H- and ^{14}C-labeling were performed on separate aliquots of cells in order to ensure that no errors in counting of doubly labeled fractions had occurred. The ratios of the "relative specific activities" of IgG (^{3}H/^{14}C) in smooth and rough endoplasmic reticulum are shown in Table 2 for

TABLE 2. *Ratio of relative specific activities (^{3}H-IgG/^{14}C-IgG) of smooth to rough endoplasmic reticulum*

Experiment no.	Leucine	Galactose	Glucosamine
1	1	11.0	5.8
2	1	35.3	10.0
3*	1	56.1	4.3
4	1	17.5	5.6

* Summarized in Table 1.

each of the ^{3}H precursors in the four experiments. The ratio of smooth to rough endoplasmic reticulum with leucine as precursor was adjusted to 1 in order to facilitate comparison between the results of the four experiments.

As can be seen, there is a definite pattern in the four experiments though the smooth/rough ratios vary. Thus, the ratio with [^{3}H]galactose ranged from 11:1 to 56:1 when compared to that of leucine. The possibility that radioactive galactose in the medium had been depleted and that the preferential labeling in the smooth endoplasmic reticulum was actually the result of transport of IgG previously labeled in the rough endoplasmic reticulum was considered. This was excluded, however, because at the conclusion of the experiment the medium was still capable of effectively labeling IgG in new P_3K cells; similar results were obtained for medium from the [^{3}H]leucine and [^{3}H]glucosamine experiments.

Percentage of radioactive IgG in microsomal supernatants: This percentage

is shown for each ^3H precursor in the four experiments summarized above. As can be seen in Table 3, a minor portion of radioactivity was found in the microsomal supernatant. If radioactive IgG in the nuclear pellet (mainly if not exclusively within microsomes) is taken into account, the percentages become even smaller.

TABLE 3. *Percentage distribution of ^3H-labeled IgG between microsomal supernatant and microsomes.**

Experiment no.	^3H-Isotope		
	Leucine	Galactose	Glucosamine
1	34	12	6
2	21	38	39
3	8	14	13
4	7	25	17

$$\text{* Percentage} = \frac{\text{Microsomal supernatant}}{\text{RER + SER + misc. + mitochondria + microsomal supernatant.}}$$

In three of four experiments the percentages for [^3H]glucosamine and [^3H]-galactose-labeled IgG in the microsomal supernatant were higher than those of [^3H]leucine. This could be explained if the vesicles of the Golgi or the postulated transport vesicles between the Golgi and the plasma membrane[4] are more fragile (or less self-healing) than membranes of the rough endoplasmic reticulum; alternatively the IgG may be in the cell cytoplasm at this late stage.

Chase experiments of [^3H]-valine-labeled IgG: In order to determine the early step in intracellular transport, P_3K cells were exposed to [^3H]valine for 2 min and then to 200× unlabeled valine. Further, to ensure the effectiveness of the chase, cycloheximide was added 8 min after the addition of the unlabeled valine. Presumably at that time all labeled nascent chains had been released. Aliquots of the cells were obtained at 2, 8, 45, and 90 min after the chase, and were fractionated as above. Attempts to perform this experiment with double labels were unsuccessful for technical reasons.[12]

As can be seen in Table 4, the valine-labeled IgG increased in the rough en-

TABLE 4. *Distribution of IgG labeled with [^3H]valine after chase with unlabeled valine.*

Time after chase (min)	Radioactive IgG		
	RER (cpm)	SER (cpm)	Microsomal supernatant (cpm)
2	17,894	470	9,650
8*	21,244	885	11,650
45	15,851	1,252	9,300
90	11,960	834	9,950

* Cycloheximide (100 μg/ml) added.

doplasmic reticulum between 2 and 8 min after chase, not because of ineffectiveness of chase (preliminary experiments proved that the valine chase was successful), but because of continued entrance of labeled nascent chains into antigenically recognizable IgG. Over a period of 90 min, approximately one half of the radioactive IgG was chased out of rough endoplasmic reticulum. In contrast, labeled IgG increased in the smooth endoplasmic reticulum between

2–8 and 45 min. By 90 min, at which time a considerable amount of secretion had taken place, radioactive IgG was decreased in both microsomal fractions. There was no significant change in the amounts of radioactive IgG found in the microsomal supernatants.

These findings support the idea that the polypeptide chains are synthesized in the rough endoplasmic reticulum and that the route of transport of IgG is from rough endoplasmic reticulum to Golgi complex in accord with previous studies.[13,3]

Discussion. Subcellular fractionation studies of an established myeloma cell line (P_3K) that secretes IgG have further elucidated the sites of incorporation of sugars and amino acids. The results indicate that after a 2-min pulse with [^{3}H]valine, its incorporation into IgG takes place primarily in the rough endoplasmic reticulum. Subsequently, radioactive IgG can be "chased" from the rough into the smooth endoplasmic reticulum (which contains elements of the Golgi complex). A 1-hr labeling period with [^{3}H]leucine uniformly labels both microsomal compartments and shows that approximately 95% of radioactive IgG in the microsomal compartment is in the rough endoplasmic reticulum. In contrast, 1-hr labeling with [^{3}H]galactose shows more radioactive IgG in the smooth than in the rough endoplasmic reticulum. The difference in ratio between relative specific activities (^{3}H/^{14}C) of IgG in smooth compared to rough endoplasmic reticulum between galactose and leucine ranged from 11:1 to 56:1 for four experiments. A certain amount of IgG labeled with [^{3}H]galactose was found in the rough endoplasmic reticulum fraction, however. This finding most likely results from contamination of rough with smooth endoplasmic reticulum or from incorporation of galactose in the transitional elements of the endoplasmic reticulum.[13] Thus, incorporation of galactose might begin in the transitional elements and continue in the Golgi complex. The finding that the ratio with [^{3}H]-glucosamine varied from 4.3–10 compared to 1 for leucine is consistent with the idea that glucosamine is incorporated both in rough endoplasmic reticulum and in the Golgi complex. These results are in complete agreement with those obtained previously with LPC_1 cells in which three other experimental approaches were used: electronmicroscopic autoradiography,[3] the effect of puromycin on the incorporation of sugars,[2] and the rate at which radioactive IgG is secreted after labeling with sugars as compared to labeling with amino acids.[2]

Our fractionation studies also showed that most of the labeled IgG is found in the microsomes regardless of precursor used. The cause of the difference between these results and those of Swenson and Kern is not clear. The initial steps in fractionation appear quite similar, i.e., use of 0.88 M sucrose and homogenization. In contrast to these authors, we used double labels in order to avoid possible variations between different aliquots of cells or different experiments. Although the cell type involved was different, we would not expect major differences in the fundamental subcellular events underlying incorporation of sugars or the intracellular route of transport between mammalian lymphoid cells, including myeloma cells.

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[10] 5 g 2,5-diphenyloxazole (PPO/liter toluene).

[11] 100 g naphthalene and 5 g 2,5-diphenyloxazole/liter dioxane. Liquid Scintillation Systems Manual 1967, Beckman Instruments, Inc., Fullerton, Calif.

[12] It was necessary that the rate of IgG synthesis by P_3K cells remain unchanged for a 3-hr period because exposure to ^{14}C-label had to precede that by ^{3}H-label by 90 min in order to label intracellular IgG uniformly. Moreover, ^{14}C-label had then to be continued in order to avoid loss of uniformity of labeling. Therefore, the 2-min labeling period with [^{3}H]valine was planned to occur at *different* times after the cells were incubated so that all cell aliquots were incubated for the same 3-hr period regardless of duration of chase. However, the rate of IgG synthesis fell off slightly but progressively during the 3 hr of incubation.

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