BIOSYNTHESIS OF GLOBOSIDE AND FORSSMAN-RELATED GLYCOSPHINGOLIPID IN MOUSE ADRENAL Y-1 TUMOR CELLS*

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SUMMARY - The activities of four glycolipid glycosyltransferases involved in the biosynthesis *in vitro* of globoside and Forssman hapten were measured in normal mouse adrenal tissues, Y-1 mouse adrenal tumor cells, and tumors derived therefrom. These enzyme activities, found in Golgi-rich membranes isolated on a sucrose density gradient, were higher in the tumor cell cultures than in normal mouse adrenal tissue. The ratio between long chain oligoglycosylceramides and short chain glycosphingolipids was higher in the case of Y-1-K cells treated with dibutyryl cAMP than in concanavalin A, colchicine treated, or untreated control cells in culture.

The chemistry, metabolism and functional role of the neutral glycosphingolipids found in animal cell membranes have been of great interest in recent years. These complex macromolecules which confer unique antigenic properties to the cell have been proposed to play an important role in intercellular recognition and density-dependent inhibition of growth (1-3).

A triglycosylceramide (Galα1-4Galβ1-4Glc-cer, GloboTri-cer) and a tetraglycosylceramide (GalNAcβ1-3Galα1-4Galβ1-4Glc-cer, globoside) were isolated from various animal cell membranes including human erythrocytes (4-6). Laine *et al* (7) and Siddiqui *et al* (8) reported the isolation of a globoside-related glycolipid from murine tissues with a difference only in the internal core α-galactose linkage. It was reported (9,10) that the internal core structure of equine spleen Forssman hapten is similar to that of the human erythrocyte globoside. Mallette and Rush (11) on the other hand suggested a globoside-like structure with terminal α-linkage for the Forssman hapten of sheep erythrocytes.

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Previously we reported the biosynthesis *in vitro* of globoside, Forssman and blood group A-related glycosphingolipids in guinea pig bone marrow (12) and embryonic chicken brain (13). Biosynthesis of these glycosphingolipids has also been shown recently in normal guinea pig kidney membranes (14,15). Incorporation of [1-^{14}C] palmitate into all these glycolipids has been studied recently by Critchley and Macpherson (16), using NIL-2-hamster cell culture. Sakiyama and Robbins (17) and Hakomori and Kijimoto (18) found that the net synthesis and amount of Forssman hapten increased at confluency in NIL-2-hamster cells. Forssman hapten was also found in metastatic tumors of human liver by Kawanami (19).

The present studies are concerned with the detection and characterization of the following four glycolipid glycosyltransferases in mouse adrenal tumor cells:

Glc-cer $\xrightarrow{\text{UDP-Gal}}$ Galβ-Glc-cer $\xrightarrow{\text{UDP-Gal}}$ Galα-Galβ-Glc-cer $\xrightarrow{\text{UDP-GalNAc}}$

GalNAcβ-Galα-Galβ-Glc-cer $\xrightarrow{\text{UDP-GalNAc}}$ GalNAcα-GalNAcβ-Galα-Galβ-Glc-cer

MATERIALS AND METHODS

Cell culture: Clone Y-1-K was isolated by the single cell plating method of Puck *et al* (20) from the Y-1 cell line originally described by Yasumura *et al* (21). Retention of the differentiated state of this cell line was evidenced by the production of 3,4-ketosteroids upon ACTH stimulation. The standard medium was F-12 (Gibco) supplemented with 15% horse serum (Gibco), 2% fetal calf serum (Gibco), penicillin (100 units/ml) and streptomycin sulfate (100 μg/ml). Cultures were grown in 250 ml Falcon flasks containing 12 ml of medium in a humidified atmosphere of 95% air, 5% CO_2. Medium was changed twice a week, and subcultures were made when the monolayers reached confluency, using 0.2% trypsin or 0.25% Viokase in phosphate-buffered saline (PBS).

Tumor growth: The Y-1 cell line, when injected into LAF_1/J mice (The Jackson Laboratory, Bar Harbor), gave rise to malignant tumors which were excised when they attained a diameter of 1-2 cm.

Preparation of membrane fraction: The enzymes which catalyze the biosynthesis *in vitro* of lactosylceramide, GloboTri-cer, globoside, and Forssman hapten were present in a membrane fraction isolated from tumors, uncloned Y-1 cells and cloned Y-1-K cells. The Golgi-rich membrane fractions were isolated from the Y-1 tumor cells according to a modification of the method of Keenan *et al* (22). Tumors were homogenized for two 15 sec periods in 2-3 volumes of 0.5 M sucrose (1.0% dextran, 0.05 M Tris-maleate, pH 6.4) with a Polytron 10-ST homogenizer. The homogenate was applied to a discontinuous gradient of 0.8 M and 1.2 M sucrose and centrifuged for 1 hr at 52,000 x g in an SW 27 swinging bucket rotor in a Beckman L2-65B preparative ultracentrifuge. The membrane fraction concentrated at the junction of 0.8 M and 1.2 M sucrose contained 50-70% of the total enzyme activity present in the homogenate. The membrane layer was separated and diluted with distilled water, pelleted at 105,000 x g for 1 hr and resuspended in 0.32 M sucrose containing 0.001 M EDTA and 0.01 M 2-mercaptoethanol to a final protein concentration of 3-5 mg of protein per ml. In the case of normal adrenal tissue, Y-1 uncloned cultures, and cloned Y-1-K cultures unfractionated homogenates (in 0.5 M sucrose) were used as the enzyme source.

Acceptors: Lactosylceramide and GM3 ganglioside were isolated from bovine spleen. GloboTri-cer and globoside were isolated from porcine heart and porcine erythrocytes according to the method of Chien *et al* (13). LacnTet-cer (Galβ1-4GlcNACβ1-3Galβ1-4Glc-cer) was prepared from rabbit erythrocyte B-active pentaglycosylceramide either by controlled periodate oxidation (24) or by treatment with fig α-galactosidase (25). Forssman hapten (glycosphingolipid) was isolated from sheep erythrocytes and purified according to the method followed for rabbit erythrocyte glycolipid isolation (26). Purified Forssman hapten from sheep erythrocytes gave a single precipitin line against anti-Forssman glycolipid rabbit antiserum. The purified glycosphingolipids were analyzed before use as substrates by gas chromatography (24,26) and by GC-mass spectrometry (27). Professor Charles C. Sweeley

Acceptor	[^{14}C] galactose incorporated (pmoles/mg protein/30 min)			
	Normal Adrenal	Y-1 Tumor	Y-1 Culture	Y-1-K Culture
None (Endogenous)*	46	35	29	303
Glc-cer (0.8 mM)	132	61	67	145
Lac-cer (2.0 mM)	43	33	405	1173
Lac-nTet-cer (1.0 mM)	125	75	73	629

Table I. Glycolipid:galactosyltransferase activities. The complete incubation mixtures contained the following components (in micromoles) in a final volume of 0.05 ml:acceptor lipids, 0.04 - 0.1; Triton X-100, 150 µg; cacodylate-HCl buffer, pH 7.25, 10; $MnCl_2$, 0.5; UDP-[^{14}C]-Gal (1.9×10^6 cpm/µmole), 0.02; and enzyme fraction, 0.25 mg of protein. The mixtures were incubated for 30 min at 37°, and the reactions were stopped by adding 2.5 µmoles of EDTA (pH 7.0). The incorporation of radioactive glycose units into acceptor glycosphingolipids was assayed by one of the following procedures. Biosynthesis of mono- to triglycosylceramide was assayed by the silica gel SG-81 paper chromatographic technique (23). Studies on the biosynthesis of tetra- and pentaglycosylceramides were made using the double chromatographic technique (13). A combination of high-voltage borate electrophoresis and reverse-flow chromatography on Whatman 3MM (24) was also used to assay some of the galactosyltransferase activities. Under these conditions reaction rates remained constant with incubation time up to 30 minutes and were proportional to protein concentration between 0.1 and 0.6 mg/0.05 ml of incubation volume.
* [^{14}C] galactose incorporated into total unidentified glycolipids.

of Michigan State University kindly provided the use of an LKB 9000 GC-mass spectrometer for analytical work. Mass spectral data were interpreted according to Bjorndal *et al* (27) and Sung *et al* (28).

Donors: Unlabelled UDP-galactose was purchased from Calbiochem and unlabelled UDP-GalNAc was prepared according to the method of Carlson *et al* (29). UDP-[^{14}C] galactose (274 mCi/mmole) and UDP-N-acetyl-[1-^{14}C] galactosamine (49.5 mCi/mmole) were purchased from New England Nuclear.

Anti-sera: Anti-Forssman rabbit antiserum was a gift from Dr. S. Hakomori. Anti-globoside rabbit antiserum was prepared according to the method of Hakomori *et al* (6). The specific antisera were used to characterize the substrate globoside and the products, [^{14}C] globoside and [^{14}C] Forssman hapten, using microimmunodiffusion (13) and microprecipitation (14) techniques.

RESULTS AND DISCUSSION

The activity of UDP-Gal:glucosylceramide β-galactosyltransferase was

Acceptor	[^{14}C]GalNAc Incorporated (pmoles/mg protein/30 min)			
	Normal Adrenal	Y-1 Tumor	Y-1 Culture	Y-1-K Culture
None (endogenous)	12	25	2	0
Lac-cer (bovine spleen)	0	41	38	8
GloboTri-cer (bovine RBC)	39	148	479	584
GloboTri-cer (porcine RBC)	52	111	386	361
GloboTri-cer (rabbit RBC)	97	120	416	ND*
GM3 ganglioside (bovine spleen)	0	53	114	33
Globoside (porcine RBC)	0	ND	29	33
Lac-nTet-cer (rabbit RBC)	0	12	16	29

Table II. Glycolipid:N-acetylgalactosaminyltransferase activities. The complete incubation mixture contained the following components (in micromoles) in a final volume of 0.05 ml: acceptor lipids, 0.05; Na-taurocholate (Sigma), 125 µg; MES buffer, pH 6.38, 10; $MnCl_2$, 0.5; UDP-[^{14}C]GalNAc (1.75 x 10^6 cpm per µmole), 0.02; and enzyme fraction 0.25 mg of protein. The mixtures were incubated for 30 minutes assayed by the double-chromatographic method (13). *ND, not done.

Agents	Lane No. in Fig. 1	Concentration	Ratio of [^{14}C]-GM3, penta-cer and tetra-cer to [^{14}C]-mono-cer plus di-cer
None	4	-	2.7
Concanavalin A	1	33 µg/ml	2.4
$(But)_2$ cAMP	2	1 mM	3.9
Colchicine	3	0.1 mM	2.3
ACTH	5	2 mU/ml	2.5

Table III. Incorporation of [^{14}C] galactose into glycolipids of Y-1-K cultures. [^{14}C] galactose (3 µCi; 7 µCi/µmole) was added to confluent Y-1-K cell cultures (0.5 - 0.8 x 10^7 cells/75 cm^2 in 250 ml Falcon flasks) and incubated for 24 hr in the presence of the indicated chemicals added to 12 ml of F-12 medium. Cells were harvested with 3 ml of 0.2% trypsin in PBS. Incorporation of [^{14}C] galactose into different glycosphingolipids was determined qualitatively on silica gel G thin layer plates (Fig. 1) and quantitatively by SG-81 paper chromatography (13,23). The ratio was determined from the quantitative assay after proper identification of individual [^{14}C] glycolipids.

determined in homogenates of normal adrenal tissue, Y-1 tumors, uncloned Y-1 cells, and cloned Y-1-K cells (Table I). [^{14}C] lactosylceramide was treated with papaya β-galactosidase which cleaved 60% of the terminal [^{14}C] galactose

suggesting that the terminal linkage was primarily in the β configuration.

A high level of activity of UDP-Gal:lactosylceramide α-galactosyltransferase was found in the Y-1 and Y-1-K homogenates whereas this enzyme was significantly lower in homogenates of normal adrenal tissue and transplantable Y-1 tumors (Table I). Whether the differences in these enzyme activities were due to the presence of some specific inhibitors, or because of an actual difference in these enzyme levels is not known at present. The product of this reaction, [^{14}C] GloboTri-cer, was purified and the terminal galactose linkage shown to be predominantly in the α configuration by its ability to be cleaved by fig α-galactosidase (24,25). This enzyme cleaved 78% of the terminal [^{14}C] galactose of the [^{14}C] GloboTri-cer. [^{14}C] GloboTri-cer and [^{14}C] lactosylceramide migrated on silica gel G thin-layer chromatograms (chloroform-methanol-water, 60:30:6, Solvent I) with authentic GloboTri-cer (Galα1-4Galβ1-4Glc-cer) and lactosylceramide (Galβ1-4Glc-cer), respectively.

Significant galactosyltransferase activity was also observed with Lac-nTet-cer as acceptor, and the [^{14}C] product co-chromatographed on SG-81 paper with rabbit blood group B pentaglycosylceramide. Biosynthesis of this blood group B rabbit-type pentaglycosylceramide may have been due to non-specific transfer of α-galactose to Lac-nTet-cer (Table I) by the same α-galactosyltransferase which catalyzes the synthesis of GloboTri-cer.

N-acetylgalactosaminyltransferase activities of normal adrenal, uncloned Y-1, and cloned Y-1-K homogenates were examined using various acceptor molecules (Table II). The GloboTri-cer (Galα-Galβ-Glc-cer) acceptors exhibited similar activities regardless of their source. This pattern of lipid acceptor specificity differs from that previously described for N-acetylgalactosaminyltransferase of embryonic chicken brain (13,30). It also appears that GM3 and GloboTri-cer are utilized as acceptors by two different N-acetylgalactosaminyltransferases present in mouse adrenal tumor cells. The purified [^{14}C] globoside product co-chromatographed with authentic porcine erythrocyte globoside (GalNAcβ1-3Galα1-4Galβ1-4-Glc-cer), and the terminal

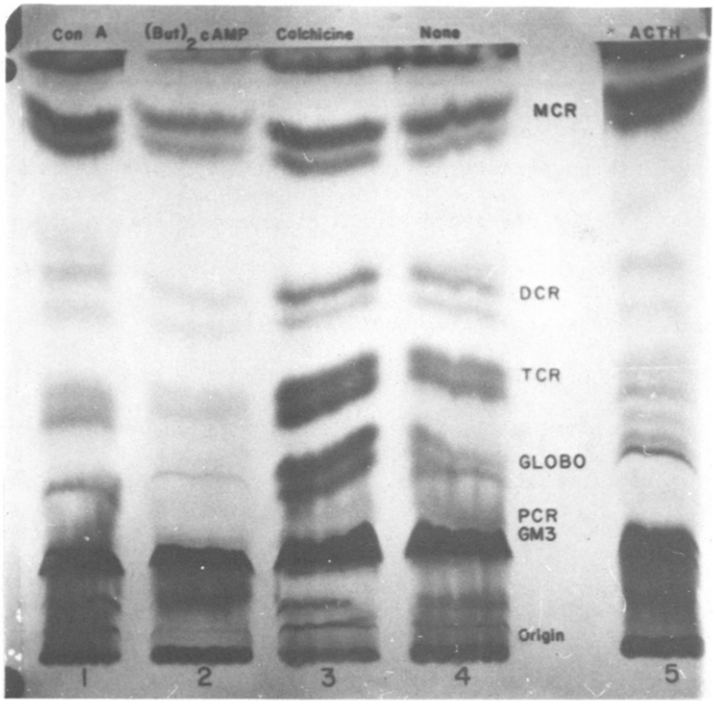

Figure 1. Radioautogram of [^{14}C] glycolipids: 10,000 dpm/sample was applied to silica gel G plate and developed with chloroform-methanol-water, 60:30:6; x-ray plate was exposed for 21 days. CMR = monoglycosylceramide; CDR = diglycosylceramide; TCR = triglycosylceramide; Globo = globoside; PCR = pentaglycosylceramide; GM3 = ganglioside (NAN-Gal-Glc-cer).

[^{14}C]GalNAc was cleaved (80%) by the action of pure jack bean β-hexosaminidase (6), confirming the β configuration of the terminal linkage of this product.

The activity observed for the biosynthesis *in vitro* of Forssman hapten from globoside (Table II) was low, but is considered significant in view of the specificity of the assay. [^{14}C] product (PCR, Fig. 1) isolated from the cell culture experiment (Table III) co-chromatographed on silica gel G thin layer plate (developed two dimensional, solvent I and n-propanol-H_2O = 7:3) with authentic sheep erythrocyte Forssman hapten. The specificity of the immunoprecipitation reaction of [^{14}C] product against anti-Forssman antiserum provides further support for the synthesis of Forssman hapten in the adrenal tumor cells. Cleavage of 85% of the terminal N-acetylgalactosamine of the [^{14}C] pentaglycosylceramide by porcine α-hexosaminidase is also consistent

with its identification as Forssman hapten. Further characterization to elucidate the chemical structure of Forssman hapten from Y-1-K cells is under investigation.

Although Forssman glycolipid has not been previously isolated from normal mouse tissues, mouse is reported to be immunologically Forssman positive (31). It has been reported in the BHK cell line (32), embryonic chicken fibroblasts (33), and NIL-2-hamster cell line (17,18) that the amount of Forssman-type complex glycosphingolipids increased when cell cultures reached confluence. While we have no evidence for such a phenomena in mouse adrenal tumor cells, we did investigate the effect of several "membrane active" factors (dibutyryl cyclic AMP, concanavalin A, colchicine, and ACTH) on the pattern of glycosphingolipid biosynthesis in these cultures (Table III, Figure 1). The increase in the ratio of tetra-cer, penta-cer and GM3 to di-cer and mono-cer in cultures incubated for 24 hours in the presence of dibutyryl cyclic AMP suggests the possibility that cyclic AMP is involved in the regulation of the metabolism of adrenal tumor cell membrane components. Whether this is related to density-dependent inhibition of growth such as reported by Hsie and Puck (34) for Chinese hamster ovary cells remains to be investigated.

Colchicine, which prevents cells from dividing by blocking them in M phase, did not alter the ratio of long chain to short chain glycosphingolipids, however, it did stimulate the synthesis of all of the intermediate glycosphingolipids necessary for the *de novo* synthesis of globoside and Forssman hapten (Figure 1). No explanation of this finding is yet available. The relationship between the morphological changes (rounding up) of the adrenal tumor cells in the presence of either ACTH or cyclic AMP and the biosynthesis of specific membrane components, including long chain glycosphingolipids is currently being investigated.

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