ENZYMIC CONVERSION OF "H_1-GLYCOLIPID" TO A OR B-GLYCOLIPID AND DEFICIENCY OF THESE ENZYME ACTIVITIES IN ADENOCARCINOMA*

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Summary - "H_1-glycolipid" (αL-Fucl→2βGall→4βGlcNAcl→3βGall→4Glc→Ceramide) was converted to "A^a-glycolipid" (αL-Fucl→2{αGalNAcl→3}βGall→4βGlcNAcl→3 βGall→4Glc→Ceramide) by N-acetylgalactosaminyl transferase of A serum or that prepared from gastrointestinal mucosal epithelia. Conversion of H_1-glycolipid into B-I glycolipid (αL-Fucl→2{αGall→3}βGall→4βGlcNAcl→3βGall→4 Glc→Ceramide) was observed by galactosyl transferase of gastrointestinal mucosal epithelia. The activities of these enzymes in carcinoma derived from gastrointestinal epithelia were found to be only 1/5 to 1/6 of the normal value.

Fucosphingolipids have been isolated from erythrocyte membranes (1-3), gastrointestinal mucosa (4), pancreas (5), and tumors of gastrointestinal tract (6,7). They constitute an essential part of membrane-bound blood group ABH and Lewis antigens (2,3,5), besides carrying unknown specificity (7), although part of blood group A and B antigens could be carried by "glycophorin" protein (8). Plasma membrane of mucosal epithelia (microvillus and brush border membrane) has a very high content of glycolipids and fucolipids (9).

Enzymatic conversion of "lacto-N-fucopentaose I" or α-L-fucosido(1→2)-lactose into A-active or B-active oligosaccharides has been demonstrated by enzymes of milk (10), stomach mucosa (11), and serum (12,13). A similar conversion of "H_1-glycolipid" (lacto-N-fucopentaosyl-IV-ceramide) (2c) into "A^a-glycolipid" (2d) or "B-I glycolipid" (2a,3,5) is now demonstrated by enzymes of serum and gastrointestinal mucosa.

Previously, we demonstrated deletion of blood group A and B activities in glycolipids with an accumulation of ceramide pentasaccharide showing H

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TABLE I. Conditions and Factors for Conversion of "H_1-Glycolipid" into "A^a-Glycolipid" or "B-I Glycolipid"

	Yield of ^{14}C-Glycolipid Product		
Conversion reaction	H_1 to A^a	H_1 to B-I	H_1 to A^a
Enzyme source (blood type)	*cecal mucosal epithelia(A_1)*	*gastric mucosal epithelia(A_1,B)*	*serum (A_1)*
Unit of activity	CPMx10^{-3} per 300 µg of "P2+3" protein per hour		CPMx10^{-3} per 60 µl serum per hour
*COMPLETE SYSTEM**	71.1	51.0	8.3
INCOMPLETE SYSTEM			
-"Cutscum"	50.5	-	-
-ATP	34.8	25.2	4.7
-Mn^{++}	0.03	0.02	0.05
-enzyme	0.10	0.10	0.03
-H_1 glycolipid	0.41	0.30	0.04
-H_1 glycolipid but + other glycolipid†	0.25-0.33		
-UDP-GalNAc +UDP-GlcNAc	0.22		

* A mixture containing the following components was prepared in a final volume of 100 µl: 45 µg of H_1-glycolipid (=30 *n*moles), and 0.4 mg "Cutscum" (isooctylphenoxypolyoxyethanol, Fischer Scientific) was dissolved in 10 µl of 1 M cacodylate acetate buffer and 10 µl of 0.4 M solution of $MnCl_2$. The solution was sonicated in a "Balsonic cleaning bath" to ensure complete dissolution, then were added 10 µl of 0.01 M adenosine triphosphate (ATP) and 1.15 *n*moles of UDP-N-acetyl(1-^{14}C)D-galactosamine (90,000 cpm) or UDP-(1-^{14}C)galactose (90,000 cpm), and 60 µl of enzyme (=300 µg protein). The mixture was incubated for one hour. After the reaction, 10 µl of 0.25 M EDTA and 10 µl of 0.5 M KCl was added and extracted with 0.6 ml of chloroform-methanol(2:1). The glycolipid was separated by chromatography on a thin-layer plate (Silica gel G) or Whatman SG81 as described in the text. The radioactivities of spots corresponding to authentic A^a-glycolipid or B-I glycolipid were determined by a scintillation counter. † Lactosylceramide, αGal(1→4)βGal(1→4)Glc→Ceramide, globoside, lactoneotetraosylceramide or a new ceramide pentasaccharide αGal(1→3)βGal(1→4)βGlcNAc(1→3)βGal(1→4)βGlc→Ceramide (Stellner & Hakomori, unpublished).

and Le^a activities (2b,14) and a positional isomer of Le^a (7). Consequently, enzyme activity of a particulate fraction of mucosal epithelia was compared with the same fraction of adenocarcinoma derived from mucosal epithelia. The enzymes of tumor tissue displayed only 1/5 to 1/6 of the activity of the host's normal mucosal epithelia, which is also reported in this communication.

MATERIALS AND METHODS

Tissues and Serum: Sera of A_1 and A_2 individuals were obtained from volunteers and were used for enzyme analysis immediately. Adenocarcinoma tissue,

freed from necrotic tissue, and the normal part of mucosal epithelia, freed by careful scraping with a special blade, were obtained from surgical or necropsy samples of the same individuals for comparison. The tissues were immediately frozen in dry ice and stored at -90°C until use. Four surgical cases and five necropsy cases were analyzed for enzyme and immunological assays.

Preparation of Enzyme Fraction: The tissues (2g) were homogenized in 5 ml of "Medium A" of Basu et al (15) that contained 0.32 M sucrose containing 0.001 M EDTA and 0.014 M mercaptoethanol at pH 7.0 and 0.3% "Triton X100" (Rohm and Haas, polyoxyethylene ether); they were homogenized in an "Omnimixer" (Sorvall, Norwalk, Conn.) for 30 seconds followed by 90 second sonication with "Biosonic III" (Bronwill Scientific, Rochester, N.Y.) using a "needle probe" (12.5x0.25cm) in an ice-water bath. Output intensity was set at 60 watts (dial at "20"). The homogenate was filtered through two layers of gauze and centrifuged at 2,000 x g for 20 minutes (pellet called P1). The supernatant was then centrifuged in a Sorvall centrifuge at 12,000 x g for 20 minutes (pellet called P2). The second supernatant was then centrifuged in a Spinco model L at 105,000 x g for one hour. The transferase activities were found in the pellet (called P3). Alternatively, the supernatant of "P1" was directly centrifuged in a Spinco L at 105,000 x g to obtain pellet P2+3. The P3 or P2+3 fraction was suspended in "Medium A" of Basu et al (15) by the aid of a microhomogenizer. The protein content of the enzyme suspension was 0.5mg/0.1 ml. A brief sonication as described above was essential for demonstrating high activity. Comparison of enzyme activities of different samples was based on protein content. Both adenocarcinoma and mucosal epithelia with the same amount of deoxyribonucleic acid gave "P2+3" fraction containing approximately the same amount of protein.

Substrate Glycolipid and Enzyme Assay: "H_1-glycolipid", "A^a-glycolipid", "B-I glycolipid", and other glycolipids were prepared from erythrocyte membrane as described previously (2-5). The conditions for enzyme reactions demonstrating the highest activity were obtained after many trials, as described in Table I and the footnote. Cold UDP-GalNAc was kindly donated by Dr. S. Basu, Notre Dame University.

Characterization of Biosynthesized Product: The biosynthesized product was isolated from the chloroform-methanol extract of the reaction mixture by thin-layer chromatography on Silica gel H with chloroform-methanol-water (60:30:8) and by paper chromatography on Silica gel impregnated paper (Whatman SG81) with chloroform-methanol-water (60:20:2). The radioactive reaction product was mixed with 100 µg of cold "A^a-glycolipid" dissolved in 100 µl of water, and 20 µl aliquots were reacted with purified Dolichos biflorus lectin, kindly donated by Dr. M. Etzler, University of California at Davis, on a thin-layer of 0.5% agarose using a special template (see 5). After the precipitin line was formed, the gel layer was washed with salt solution followed by distilled water, stained, and dried. The stained, dried gel with precipitin line was then exposed on X-ray film (exposure time varied depending on experiments, 2 weeks to 2 months). The radioactive sugar components in the biosynthesized product was examined by paper chromatography (Whatman 3MM, ethyl acetate-pyridine-water, 12:5:4) after hydrolysis (3 N HCl, 100°C, 4 hours), followed by scanning with strip scanner (Packard).

RESULTS

As seen in Table I, a very effective conversion of H_1-glycolipid into A^a-glycolipid was seen in the presence of adenosine triphosphate, "Cutscum", and Mn^{++}. These conditions are similar to those described by Kim et al (12)

TABLE II. Conversion Rate of H_1 into A^a Glycolipid by Enzymes from Various Sources: Yield of A^a-Glycolipid in CPMx10^{-3} and % Conversion Expressed by Added UDP-Sugars

		Incorporation of ^{14}C-GalNAc into A^a-Glycolipid		
Enzymes of:	Donor	Blood Group	CPMx10^{-3}	% Added Radioactivity
Serum	C.Ga	A_1	8.33	9.2
	R.Je	A_1	9.83	10.9
	N.Ga	A_2	0.24	0.2
	J.Ca	A_2	0.23	0.2
	G.Yo	B	0.04	0.04
	K.St	O	0.03	0.03
Tumor from mucosal epithelia	L.Ek	A_1	14.34	15.5
	T.Wn	AB	3.9;2.1*	4.3;2.2*
	N.Da	A_2	4.2	4.6
	A.Wm	A_1	5.1	5.6
Host's mucosal epithelia	L.Ek	A_1	71.18	79.0
	T.Wn	AB	28.17;12.2*	31.3;13.5*
	N.Da	A_2	8.64	9.6
	A.Wm	A_1	30.05	33.3

* H_1 to B conversion determined with ^{14}C-Gal under the same conditions as for H_1 to A conversion.

TABLE III. Hydrolase Activity of "P2+3" for "A^a-Glycolipid": % of Hydrolyzed Activity to Substrate Activity*

Case donor	Normal	Tumor
L.Ek	12	13
N.Da	14	12
T.Wn	12	15

*Biosynthesized radioactive A^a-glycolipid was dissolved in 50 µl of citrate buffer pH 4.5 containing µg of sodium taurocholate, and incubated with 50 µl of enzyme (Fraction "P1+2") for 16 hours at 37°C. The liberated N-acetylgalactosamine was separated on paper chromatography (solvent: ethyl-acetate-pyridine-water 12:4:4). The activity of N-acetylgalactosamine was counted and expressed as % of total radioactivity of A^a-glycolipid added.

and Schachter *et al* (13). The substrate, H_1-glycolipid, cannot be replaced by other glycolipids with other terminal structures (footnote, Table I).

The biosynthesized product showed an identical Rf value as A^a-glycolipid (2d) or B-I glycolipid (2a,3,5) by chromatographies on Silica gel H or Whatman SG81 paper, and the A^a-like product gave a radioactive precipitin line indistinguishable from the line between A^a-glycolipid and *Dolichos biflorus* lectin, and the radioactivity in the hydrolysate was detected only in the spot on paper chromatography corresponding to galactosamine.

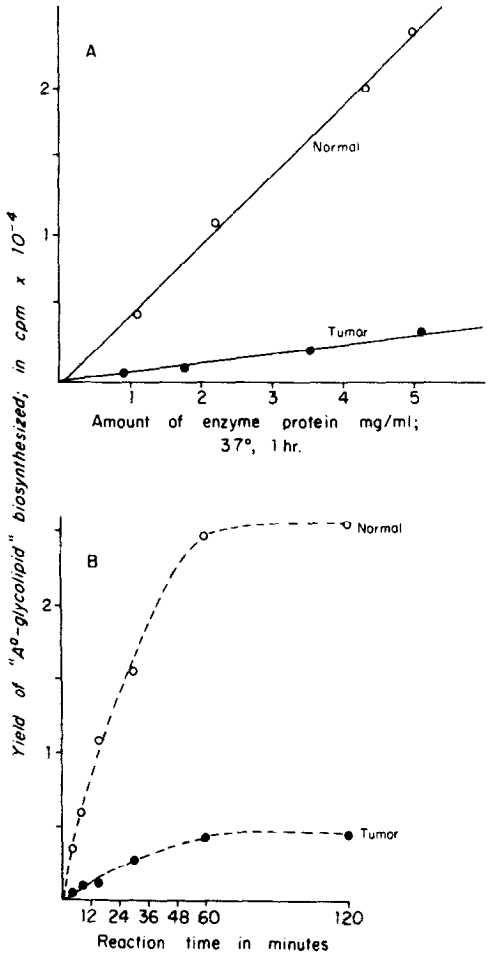

FIGURE LEGEND. Figure 1: Difference in A^a-glycolipid synthesis between "P2+3" fractions of normal gastric mucosa and tumor: difference of reactivities depending on the amount of enzyme protein (A) and on reaction time (B). Enzyme protein in "B": 5 mg/ml (300 µg/100 µl). Open circle: normal gastric mucosal epithelia; solid circle: tumor (case ot T.Wn in Table II).

A higher amount of synthesis was observed when the H_1-glycolipid/UDP-GalNAc ratio was higher, i.e. about 30% of ^{14}C-UDP-GalNAc was incorporated into A^a-glycolipid when the ratio was 25 to 1, whereas only 9% of added ^{14}C-UDP-GalNAc was incorporated into A^a-glycolipid when the ratio was 2 to 1. Addition of cold UDP-GalNAc did not increase the rate of synthesis.

As seen in Table II and Figure 1, a much higher rate of conversion took place when catalyzed by the enzyme of normal epithelial mucosa as compared to the same reaction catalyzed by the enzyme of adenocarcinoma tissue derived

from epithelial mucosa of the same individuals. The difference was also clear when the yields of radioactive A^a-glycolipid were compared by increasing the concentration of enzyme proteins or by increasing incubation time (Figure 1). The hydrolase activity for A^a-glycolipid of "P2+3" fraction of normal mucosal tissue was nearly identical to that of adenocarcinoma (Table III). Higher A^a synthesis was observed by A_1 serum than A_2 serum in agreement with Schachter et al (13).

DISCUSSION

Deletion of blood group A and B determinants in glycoproteins of adenocarcinoma has been known for two decades (16,17), although the activities of H and Lewis antigens were less impaired or even increased (14,17). It is noteworthy that diminished or totally deleted A or B reactivity accompanying malignancy were detectable by immunofluorescence or by mixed hemagglutination at the very early stage of the transformation, even at pre-malignant lesion ("dysplasia" or "atypia") (18). This study shows that deletion of A and B reactivities in epithelial tumor is due to a deficiency of glycosyltransferases for synthesis of A and B determinants but is not due to enhanced hydrolase activity.

Blocked enzyme activities for synthesis of various glycolipids have been demonstrated in various transformed cells in vitro (19). A similar enzyme block for glycoprotein synthesis was also demonstrated (20). The deficient A or B enzyme for glycolipid as described here could be the same enzyme for synthesis of A or B glycoprotein as well, because blood group glycoproteins of tumors were also shown to be deficient (16). Most recently, fucolipid changes associated with in vitro transformed cells by oncoranoviruses was clearly demonstrated by Steiner et al (21), which could be analogous to the change of blood group glycolipid synthesis in human tumors, as described in this paper.

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