BIOSYNTHESIS OF GUINEA PIG ERYTHROCYTE TRIGLYCOSYLCERAMIDE BY BONE MARROW β-N-ACETYLGALACTOSAMINYLTRANSFERASE

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SUMMARY A β-N-acetylgalactosaminyltransferase (GalNAcT) that catalyzes the synthesis of a triglycosylceramide, GanglioTri-cer (GalNAcβ-Galβ1-4Glc-cer), from lactosylceramide and UDP-GalNAc was isolated from guinea pig bone marrow. The enzyme was present in the supernatant solution obtained after homogenization of guinea pig bone marrow 12,000 x g pellet with 0.32 M sucrose containing 0.6% Triton X-100 and centrifugation at 129,000 x g. The enzyme that catalyzed the transfer of GalNAc to a tetraglycosylceramide (Lac-nTet-cer) was found in a membrane-bound fraction. The K_m values were 0.5 mM and 0.7 mM for the lactosylceramide and Lac-nTet-cer, respectively. 97.0% of the terminal [^{14}C]GalNAc was cleaved by the action of pure β-hexosaminidase from [^{14}C]triglycosylceramide.

The neutral glycosphingolipids containing N-acetylgalactosamine are found as major glycolipids in the red cell membranes of many animals. Each species has a particular glycolipid composition with a specific glycolipid as a major component. A tetraglycosylceramide, globoside (or GloboTet-cer, GalNAcβ1-3 Galα1-4Galβ1-4Glc-cer), is the major glycolipid in erythrocyte membranes of humans (1,2) and porcine (3), whereas a pentaglycosylceramide, Forssman hapten (GalNAcα1-3GalNAcβ1-3Galα1-4 Galβ1-4Glc-cer), is the predominant glycolipid in ovine (4) and caprine (5) red cells. It is interesting that the major glycolipid of guinea pig red cell membranes is a triglycosylceramide (or GanglioTri-cer, GalNAcβ1-4Galβ1-4Glc-cer (6)), instead of the

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Forssman active pentaglycosylceramide, though tissue homogenates from almost all the organs of the guinea pig cross-react with anti-Forssman antiserum (7). This paper describes the biosynthesis in vitro of the guinea pig erythrocyte triglycosylceramide (GanglioTri-cer) in the presence of a β-N-acetylgalactosaminyltransferase (GalNAcT) isolated from guinea pig bone marrow.

MATERIALS AND METHODS

Acceptors and Donors - GanglioTri-cer(GalNAcβ1-4Galβ1-4Glc-cer) was isolated from guinea pig blood (purchased from Rockland, Gilbertsville, Pa.) using the methods of Seyama and Yamakawa (6) and Chien et al. (8). Lactosylceramide and GM3 ganglioside were isolated from bovine spleen. GloboTri-cer(Galα1-4Galβ1-4Glc-cer) and GloboTet-cer (or globoside, GalNAcβ1-3Galα1-4Galβ1-4Glc-cer) were isolated from porcine erythrocytes and heart (8). Lac-nTet-cer(Galβ1-4GlcNAcβ1-3Galβ1-4Glc-cer) was prepared from rabbit erythrocytes B-active pentaglycosylceramide either by controlled periodate oxidation (9,10) or by treatment with fig α-galactosidase (11). Forssman glycolipid was isolated from sheep erythrocytes. Purified GloboTet-cer (globoside), Lac-nTet-cer, and Forssman glycolipid gave single precipitin lines against anti-globoside, anti-Type XIV pneumococcal polysaccharide, and anti-Forssman antisera, respectively. The purified glycosphingolipids were analyzed before use as substrates by gas chromatography (10,12) and by GC-mass spectrometry (13). Unlabeled UDP-GalNAc was prepared according to method of Carlson et al. (14) and UDP-N-acetyl-1-^{14}C galactosamine (61 mCi/mmole) was purchased from Amersham/Searle.

Preparation of Enzyme - 7 to 8 g of frozen guinea pig bone marrow (purchased from Rockland, Gilbertsville, Pa.) was homogenized with 3 volumes of 0.32 M sucrose containing 1.0 mM EDTA and 14 mM mer-

captoethanol, pH 7.0, in a Potter Elvehjem homogenizer with a loosely fitting Teflon pestle. The homogenate was centrifuged for 1 hour at 12,000 x g. The pink pellet and upper reddish supernatant were rehomogenized without the floating lipid layer. The mixture was recentrifuged in the Spinco centrifuge (L2-65B) for 90 min at 129,000 x g for one hour. The residue was rehomogenized with 8 ml of 0.32 M sucrose solution (Fraction R1). 4 ml of Fraction R1 were rehomogenized with an equal volume of 0.32 M sucrose solution containing 1.2% Triton X-100 (10). The mixture was centrifuged at 129,000 x g for 1 hour. The UDP-GalNAc:lactosylceramide β-N-acetylgalactosaminyltransferase(GalNAcT) was distributed as follows: 51.0% in the residue (Fraction R2) and 49.0% in the supernatant (Fraction S2) and the enzyme was precipitated (30% recovery) from the supernatant Fraction S2 upon 60% saturation with ammonium sulfate. However, almost 80% of the UDP-GalNAc:Lac-nTet-ceramide N-acetylgalactosaminyltransferase activity remained in the Fraction R2.

Assay Method - Complete incubation mixture contained the following components (in micromoles) in final volumes of 0.045 to 0.05 ml: acceptor lipids, 0.05; sodium taurocholate (Simga Chemical Co.), 100 µg; $MnCl_2$, 0.25; MES buffer, pH 6.5, 10; UDP-N-acetyl [^{14}C] GalNAc (2.1 to 4.1 x 10^6 dpm/µmole), 0.018; enzyme Fraction R2 (unless otherwise stated), 0.17 to 1.1 mg of protein (measured by the method of Lowry et al. (15)). The mixtures were incubated for 2 hours at 37°, and the reaction was stopped by adding 2.5 µmoles of EDTA (pH 7.0). The mixture was spotted on Whatman No. 3MM paper and subjected to a double chromatographic assay as described previously (8-10). The appropriate areas of each chromatogram were quantitatively determined in a Packard scintillation counter, model 3375. Under these conditions the rate of reaction

remained constant with time of incubation up to 2 hours and was proportional to protein concentration up to 1.2 mg of protein per 0.1 ml incubation volume. For further characterization, [^{14}C] products were eluted from the papers with chloroform-methanol-water (60:35:8) and purified by Unisil column chromatography and silica gel G thin-layer chromatography.

RESULTS AND DISCUSSION

As shown in Table I, the incorporation of N-acetyl[^{14}C]galactosamine from UDP-[^{14}C]GalNAc increased 7- to 8-fold in the presence of Lac-nTet-cer and lactosylceramide, respectively, compared with the absence of any substrate. The endogenous [^{14}C] product was characterized tentatively as GM2 from its migration on silica gel G TLC (chlorform-methanol-water, 60:35:8). Further

Incubation Mixture	[^{14}C]GalNAc incorporated into	
	Lactosylceramide (A)	Lac-nTet-cer (B)
	pmoles/mg protein/hour	
Complete	301	214
Minus substrate	39	32
Minus detergent	71	58
Minus $MnCl_2$	13	0
Plus EDTA (1.0 µmole)	12	0
Minus active, plus heat inactivated (5 min, 100°) enzyme	19	12

Table I. Requirements for guinea pig bone marrow N-acetylgalactosaminyltransferase. In experiment A the complete incubation mixture contained all the components as described in the text. In experiment B conditions were the same as described in the text except that Lac-nTet-cer, 0.2 µmole, was used instead of lactosylceramide. Fraction R2 was used as enzyme source.

characterization of this endogenous product is under investigation. Of the many detergents tested, the highest activity was obtained with sodium-taurocholate at a concentration of 2 mg per ml. The complete system with lactosylceramide or Lac-nTet-cer required $MnCl_2$, and EDTA completely inhibited the reactions. Substitution of either Cd^{2+} or Co^{2+} for Mn^{2+} reduced the rate by 80%.

Incorporation of [^{14}C]GalNAc into a number of different potential acceptors was studied using two different enzyme preparations (Table II), Fraction R2 (experiment A) and Fraction S2 (experiment B). It appears that incorporation of GalNAc into lactosylceramide is catalyzed by a different GalNAcT from that catalyzing incorporation with Lac-n-Tet-cer. The pattern of substrate specificity with guinea pig bone marrow enzyme preparations

Acceptors (2.0 mM)	[^{14}C]GalNAc incorporated	
	(A)	(B)
	pmoles/mg protein/hr	
Endogenous	47	27
Lac-cer(Galβ1-4Glc-cer)	493	1647
GloboTri-cer(Galα1-4Gal-Glc-cer)	127	ND*
GloboTet-cer(GalNAcβ1-3Gal-Gal-Glc-cer)	41	ND*
Lac-nTet-cer(Galβ1-4GlcNAc-Gal-Glc-cer)	367	157
GM3 (NANα2-3Galβ1-4Glc-cer)	861	1833

Table II. In experiment A conditions were the same as described in Table I, except that different potential acceptors were added instead of lactosylceramide. In experiment B conditions were the same as described in experiment A except that enzyme Fraction S2 was used instead of Fraction R2. ND*, not determined.

was quite different from that observed with embryonic chicken brain (8,16), rat brain (17-19), or guinea pig kidney (20) membrane bound GalNAcT activities. Recently we have reported (21) the presence of two GalNAcT activities in cultured mouse tumor cells, Y-1-K, which catalyze the synthesis of globoside and a pentaglycosylceramide, Forssman-related glycolipid. In all the above mentioned membrane-bound enzyme preparations activity with lactosylceramide was 85 to 90% lower than that obtained with other substrates such as GM3, GloboTri-cer, or Globoside, whereas almost equal activity was obtained with lactosylceramide and GM3 using Triton X-100 soluble guinea pig bone marrow GalNAcT. Very little activity (10 to 15%) was detected with GloboTri-cer

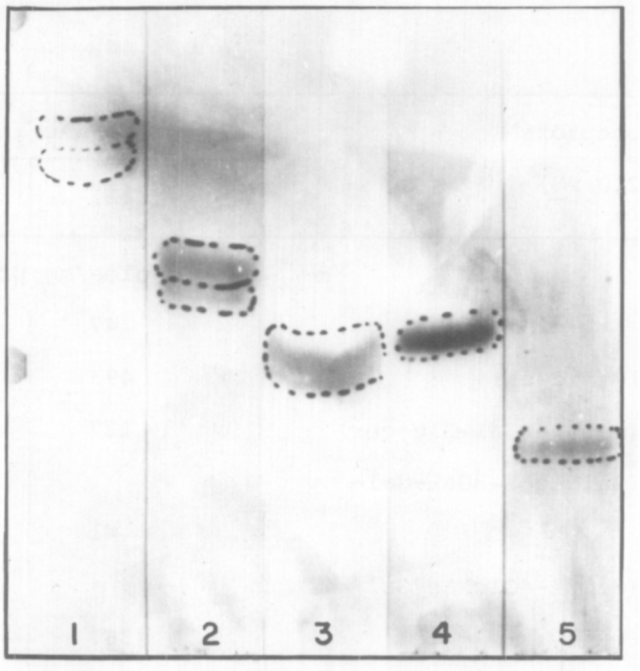

Figure 1. Radioautogram of ^{14}C-triglycosylceramide. 6200 dpms of [^{14}C] product were applied to a silica gel G plate and developed with chloroform-methanol-water, 60:30:6; the x-ray plate was exposed for 57 days. 1. Lac-cer(Galβ1-4Glc-cer); 2. GloboTri-cer(Galα1-4Galβ1-4Glc-cer); 3. [^{14}C]triglycosylceramide; 4. GanglioTri-cer(GalNAcβ1-4Galβ1-4Glc-cer); 5. Forssman hapten (GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glc-cer).

or GloboTet-cer(globoside) in this fraction, as most activity remained in the Triton X-100-nonextractable membrane preparation.

[^{14}C] product was isolated and purified by Unisil column chromatography and silica gel G thin layer chromatography using lactosylceramide as substrate. It migrated with authentic guinea pig RBC trigylcosylceramide (GanglioTri-cer) in three different solvent systems (Fig. 1). 97% of the terminal [^{14}C]GalNAc was cleaved by the action of pure jack bean β-hexosaminidase (22), confirming the β-configuration of the terminal linkage present in ^{14}C-triglycosylceramide. The ^{14}C pentaglycosylceramide obtained from Lac-nTet-cer migrated close to rabbit erythrocyte B-active pentaglycosylceramide (solvent, chloroform-methanol-water, 60:30:6), and jack bean β-hexosaminidase cleaved only 7.0% of the total radioactivity. Further characterization of this product and its relation to blood group A-active glycolipid (23,24) is under investigation.

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