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Deficiency of β1-6 N-Acetylglucosaminyltransferase Involved in the Biosynthesis of Blood Group I Antigen in the Liver of LEC Rats

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The activities of the β1-6 and β1-3 N-acetylglucosaminyltransferases, which synthesize blood group I and i antigens, respectively, were measured in various tissues of hepatitis- and hepatoma-predisposed rats (LEC rats). In LEC rats the β1-6 N-acetylglucosaminyltransferase activity was barely detectable in the liver, while substantial enzyme activity was found in other tissues. In the control LEA rats the enzyme was expressed in most tissues, including the liver. Immunochemical studies using a monoclonal antibody which recognizes I antigen indicated that the expression of I antigen was less prominent in hepatocytes of LEC rats than in hepatocytes of LEA rats. The level of β1-3 N-acetylglucosaminyltransferase activity was constant in most of the tissues during the development. These results indicate that the biosynthesis of I antigen does not occur in the livers of the LEC rats.

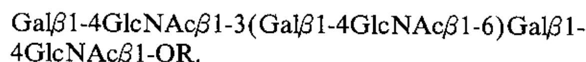
Key words: Deficiency — I antigen — Biosynthesis — β1-6 N-acetylglucosaminyltransferase — LEC rat

LEC[4] rats are a unique strain which is predisposed to developing hepatitis and hepatoma.[1-3] The genetic basis of the disease has been shown to be a single autosomal recessive mutation,[2] and an arrest of differentiation from $CD4^+8^+$ to $CD4^+8^-$ thymocytes during T cell maturation[4] and accumulation of copper in the liver as in Wilson's disease have been reported.[5] In addition, our previous studies indicated that the activities of enzymes involved in drug metabolism in LEC rat liver are very similar to those observed in precancerous regions during chemical hepatocarcinogenesis[3,6] or in choline-deficient rats.[7] Moreover, in the liver of LEC rats the pattern of glycolytic enzymes such as hexokinase is similar to those found in fetal tissues.[8] These data suggest that in LEC rats the differentiation or maturation of various cells is impaired.

It is well known that hematopoietic cells contain cell-surface carbohydrate structures which show blood group I and i activity. These antigens are differentiation antigens and are also present on the cell surfaces of many other tissues and in certain mucins.[9-12] The carbohydrate structures comprising both antigens are built from repeating units of N-acetyllactosamine (Galβ1-4GlcNAc) and are called lactosaminoglycans. The structures associated with i antigen are linear chains with N-acetyllactosamine units attached to each other in β1-3 linkage, as in the following structure:

Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-OR.

The I antigenic determinants are branched lactosaminoglycans with one or several branch points where two N-acetyllactosamine units are linked to positions 3 and 6 of a galactose, as in the following structure:


Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAcβ1-OR.

The biosynthesis of the i and I antigens is catalyzed by two glycosyltransferases, a β1-3 N-acetylglucosaminyltransferase (β1-3GnT) and a β1-6 N-acetylglucosaminyltransferase (β1-6GnT), respectively. They have been identified in hog gastric mucosa,[13] Novikoff ascites tumor cells,[14-16] human serum[17,18] and mouse T-lymphoma.[19] It has been reported that during development from fetal to adult erythrocytes, linear i-active polylactosaminoglycans are converted to branched glycans having I activity.[9,11] This convension has been proposed to be due to the action of a β1-6GnT.[9]

Our previous report indicated that in rat intestine there are two β1-6GnTs involved in the biosynthesis of I antigen.[20] One, which has been extensively studied by several authors,[13,14] transfers GlcNAc to the penultimate galactose of $LcOse_3$ and synthesizes I antigen having the structure GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4Glc. The other, a novel β1-6GnT enzyme, transfers GlcNAc to carbon 6 of internal galactose of $nLcOse_4$ and synthesizes

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[4] Abbreviations: LEC, Long-Evans with a cinnamon-like coat color; LEA, Long-Evans with an agouti coat color; GnT, N-acetylglucosaminyltransferase; PA, 2-aminopyridine; HPLC, high-performance liquid chromatography; $nLcOse_4$, lacto-N-neotetraose, Galβ1-4GlcNAcβ1-3Galβ14Glc; $LcOse_3$, GlcNAcβ1-3Galβ1-4Glc; MOPS, 3-(N-morpholino)propanesulfonic acid.

I antigen having the structure Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4Glc.

The aim of the present study was to investigate i/I antigen expression as differentiation antigens in LEC rats. We present evidence that in normal rat liver only the novel β1-6GnT was present, whereas in LEC rat liver the β1-6GnT activity was almost negligible and conversion from i antigen to I antigen did not occur due to this enzyme deficiency.

MATERIALS AND METHODS

Materials 2-Aminopyridine was obtained from Wako Pure Chemical Co., Ltd., Japan and recrystallized from *n*-hexane. β-Galactosidase was purchased from Seikagaku Kogyo Co. Ltd., Japan. Pyridylaminated N-acetylglucosamine was kindly supplied by Dr. S. Hase (Department of Chemistry, Osaka University College of Science). UDP-GlcNAc was a Sigma product. Lacto-N-neotetraose was purchased from Bio-carb Chemicals, Sweden. A monoclonal antibody, C6,[21] was a kind gift from Dr. Sen-itiroh Hakomori.

LEC and LEA rats LEC and LEA rats of both sexes were maintained in our department as described previously.[6,7]

Preparation of the enzyme substrates A fluorescence-labeled oligosaccharide, nLcOse$_4$-PA, was obtained from nLcOse$_4$ by means of pyridylamination as described previously.[22-24] Further purification of the substrate was done by HPLC on a TSK gel ODS-80TM colum (7.8$\times$300 mm; Tosoh) which had been equilibrated with 20 m*M* acetate buffer, pH 4.0, at a flow rate of 3.0 ml per minute. Elution of the PA-sugar chain was performed with the same buffer. LcOse$_3$-PA was prepared by digesting the nLcOse$_4$-PA with β-galactosidase. Fluorescence was detected with a fluorescence spectrophotometer (Hitachi, Model F-1000) with excitation and emission wavelengths of 320 and 400 nm, respectively.

Crude enzyme extracts Rats were killed under light ether anesthesia and various tissues were homogenized in 4 vol. of 10 m*M* Tris-HCl buffer, pH 7.4, containing 0.25 *M* sucrose with an Ultra-Turrax homogenizer (Ika-werk, Germany). After centrifugation at 900*g* for 10 min, the supernatants were collected and used as the crude enzyme preparations.

Enzyme assay The substrate (nLcOse$_4$-PA or LcOse$_3$-PA) was employed at a final concentration of 150 m*M* in β1-6GnTs or β1-3GnT activity assays. β1-3GnT activity was assayed as follows. The reaction mixture contained 400 m*M* MOPS buffer, pH 7.5, 40 m*M* UDP-GlcNAc, 40 m*M* $MnCl_2$, 200 m*M* GlcNAc, 200 m*M* galactose and 1.0% (w/v) Triton X-100. Ten microliters of 750 μM substrate was added to 25 μl of the reaction mixture and then 15 μl of enzyme solution was added and the mixture was incubated at 37°C for 4 h.

Assay of both types of β1-6GnT activity employed 400 m*M* MOPS buffer, pH 7.0 containing 40 m*M* UDP-GlcNAc, 25 m*M* EDTA, 200 m*M* GlcNAc, 200 m*M* galactose and 1.0% (w/v) Triton X-100. It should be noted that Mn^{2+} is not essential for β1-6GnT activity. To 25 μl of this solution was added 10 μl of 750 μM substrate (nLcOse$_4$-PA or LcOse$_3$-PA), followed by 15 μl of enzyme solution. The assay mixture was then incubated at 37°C for 4 h. These reactions were stopped by heating at 100°C for 2 min. The samples were then passed through a 0.22 μm Millipore filter and an aliquot of each filtrate was applied to a TSK gel ODS-80TM column (4.6$\times$250 mm). Elution was performed at 50°C with 20

Table I. β1-3 and β1-6 N-Acetylglucosaminyltransferase Activities in LEA and LEC Rats

Tissue	β1-3GnT activity (pmol/h/mg protein)		β1-6GnT activity (pmol/h/mg protein)			
			toward nLCOse$_4$-PA		toward LcOse$_3$-PA	
	LEA	LEC	LEA	LEC	LEA	LEC
Intestine	53±31	45±17	1190±450	150±26	1860±165	1864±113
Kidney	24±11	15±3	130±13	84±14		
Lung	32±8	24±11	120±24	74±17		
Stomach	54±6	33±7	100±14	34±8		
Liver	23±14	33±4	83±30	trace	ND	ND
Testis	30±4	12±1	ND	ND		
Spleen	60±7	64±6	ND	ND		
	(pmol/h/ml)		(pmol/h/ml)			
Serum	1110±250	1050±190	1550±550	540±210		

Each value is expressed as the mean±SD of 5 rats of both sexes at 12 weeks old. ND indicates below the detectable level.

m*M* acetate buffer, pH 4.0, at a flow rate of 1 ml/min and the eluates were examined with a fluorescence photometer. The amount of product was estimated from the fluorescence intensity by using pyridylaminated N-acetylglucosamine as a standard. The specific activity of the enzyme is expressed as pmol of N-acetylglucosamine transferred/h/mg protein or pmol/h/ml of serum. Protein was determined with a Bio-Rad protein assay kit using bovine serum albumin as a standard.[25]

Immunochemical staining Thin slices of liver and intestine of 10-week-old LEA and LEC rats were fixed with 4% buffered formalin and embedded in paraffin. The avidin-biotin-peroxidase complex method[26] was used for demonstration of the antigen in 4-μm-thick paraffin sections. Briefly, after blocking of endogenous peroxidase using 0.3% hydrogen peroxide in methanol, the sections were treated with sialidase (one unit/liter; Nacalai Tesque Co. Ltd., Japan) in 0.1 *M* phosphate buffer, pH 7.4, at 37°C for 4 h. The sections were reacted with monoclonal antibody C6 at 4°C for 24 h. Then sections were incubated with biotinylated anti-mouse IgM (Vector Labs, USA) at room temperature for 40 min, and avidin-biotin-peroxidase complex (Vector Labs) was applied at room temperature for 40 min. Sites of peroxidase activity were visualized with 0.02% 3,3′-diaminobenzidine-tetrahydrochloride (Dotite, Japan) containing 0.005% hydrogen peroxide. The sections were counterstained with hematoxylin. For negative controls, non-immunized mouse serum and culture medium were applied in place of the primary monoclonal antibody.

RESULTS

Distribution of β1-3GnT and β1-6GnT activities toward nLcOse$_4$-PA in various tissues of both LEA and LEC rats The β1-3GnT and β1-6GnT activities were examined in various tissues of 12-week-old LEA and LEC rats. As shown in Table I, in LEA rats, intestine, kidney, lung, liver and serum had high β1-6GnT specific activities. On the other hand, in LEC rats, substantial activity was found in the intestine, kidney, lung and stomach while only trace, if any, activity was found in the liver.

The β1-3GnT was widely distributed in various tissues of LEA and LEC rats. No significant difference in the activity of this enzyme was found between the two types of rats.

A typical elution pattern of the reaction products of β1-3GnT and β1-6GnT in both LEA and LEC rat livers is shown in Fig. 1. The reactions were carried out using liver extracts of LEA and LEC rats, and the products were identified by ^{1}H-NMR as described.[20]

Our previous reports indicated that normal rat intestine contains two kinds of β1-6GnTs.[20] In order to see whether there are two pathways for biosynthesis of I antigen in both LEA and LEC rats, the two β1-6GnTs activities were examined in livers and intestines of 12-week-old rats of both strains. As shown in Table I, intestines of LEA rats have high β1-6GnT specific activities toward LcOse$_3$-PA and nLcOse$_4$-PA. But in intestines of LEC rats, the activity toward LcOse$_3$-PA was high, while the activity toward nLcOse$_4$-PA was very low. On the other hand, no activity of β1-6GnT toward LcOse$_3$-PA was detected in adult livers of both types of rats. These findings indicate that there is only one pathway for biosynthesis of I antigen in adult livers, but two pathways exist in intestines of both types of rats.

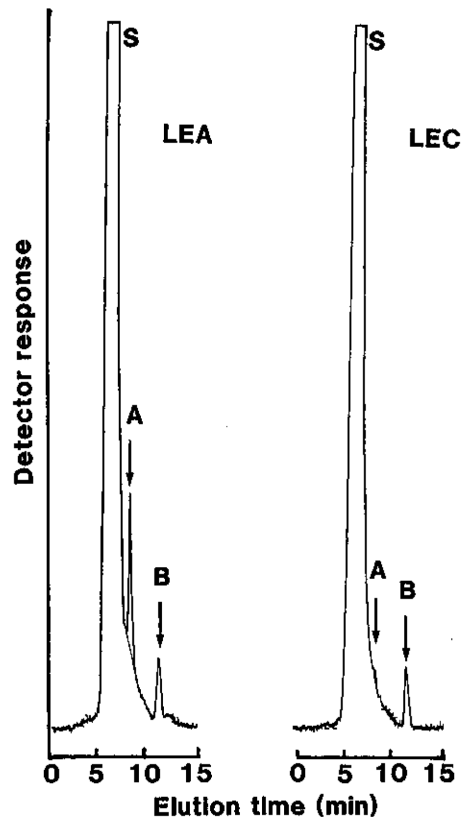

Fig. 1. A typical elution pattern of the reaction products on HPLC. Crude enzyme extract from LEC rat liver or LEA rat liver was incubated in the β1-3GnT mixture for 4 h at 37°C, and an aliquot of each was applied to an HPLC column as described under "Materials and Methods." The left panel shows an elution pattern for LEA rat liver. The right panel shows an elution pattern for LEC rat liver. The substrate (S), product (A) and product (B) were identified by comparing the elution times on HPLC with standards which had been characterized by ^{1}H-NMR. A, elution position of the reaction product of β1-6GnT that can be also identified using the assay mixture for β1-3GnT. B, elution position of the reaction product of β1-3GnT.

Table II. Developmental Changes of β1-6GnT Activity in LEA and LEC Rat Livers

Liver	β1-6GnT activity toward nLcOse$_4$-PA (pmol/h/mg protein)	
	LEA	LEC
Fetal liver		
8–10 days	57, 85 (2)	34 (2) (pooled)
Young adult liver		
2–4 weeks	49 (2)	2±1 (3)
Adult liver		
8–11 weeks	81±30 (6)	2±1 (5)
12–16 weeks	67±9 (5)	3±4 (3)
19–30 weeks	79±17 (5)	3±3 (4)
50–70 weeks	101±33 (4)	5±5 (6)

Each value is expressed as the mean±SD. Numbers in parenthesis indicate numbers of rats.

Developmental changes of β1-6GnT activity toward nLcOse$_4$-PA in LEC and LEA rat livers The levels of β1-6GnT activities in LEA and LEC rat livers at various ages were compared. Fetal rat livers of both LEA and LEC rats were found to contain approximately 34–85 pmol/h/mg proteins (Table II). The β1-6GnT activity in livers of 2- to 4-week-old LEA rats was found to be approximately 50 pmol/h/mg, whereas that of LEC rat livers was negligible. At even 50 weeks old, the β1-6GnT activity in LEC rats was barely detectable. On the other hand, no significant difference in the activities of β1-3GnT was found between the two types of rats at various ages (data not shown). These data suggest that the conversion from i to I antigen biosynthesis occurred at an early stage of embryogenesis in fetal liver, and in LEC rats the expression of β1-6GnT was impaired after birth.

Immunostaining of I antigen in liver and intestine of LEC and LEA rats In order to confirm that I antigen, a product of β1-6 GnT, was absent in LEC rat livers, immunochemical studies using a monoclonal antibody, C6, which recognizes the I antigen were carried out. In both 10-week-old LEA and LEC rats, the monoclonal antibody C6 was found to be reactive to Kupffer cells, bile duct and endothelial cells of the liver only when the sections had been treated with sialidase. No reaction was detected in any cell of the liver without sialidase treatment. The reaction was weaker in hepatocytes of LEC rats than in hepatocytes of LEA rats, as compared with the Kupffer cells, bile duct and endothelial cells, where reactions were equal in the LEA and LEC rats (Fig. 2). Because hepatocytes are the major component of the liver, the less prominent expression of I antigen in the LEC rat hepatocytes as judged by immunohistochemical staining is consistent with the biochemical findings described above. On the other hand, although β1-6GnT activity toward nLcOse$_4$-PA in LEC rat intestine is lower than that in LEA rat intestine, the intensities of immunohistochemical staining reactions were equal in LEA and LEC rats (data not shown). This result could be explained by the fact that the other β1-6GnT exists to compensate for the biosynthesis of I antigen.

DISCUSSION

The branched structure, I antigen, is known to be developmentally regulated in hematopoietic and many other tissues.[9,27,28] Until now, however, no report has been published on the expression of I/i antigens in hepatocytes. In the present study the liver of LEC rats was found to contain only a trace of β1-6GnT activity, if any. Consistent with this finding, immunochemical staining of LEC and LEA rat livers indicated that the expression of I antigen in LEC rat hepatocytes was less prominent than that in LEA rat hepatocytes. In non-parenchymatous cells such as Kupffer cells and endothelial cells of LEC rat livers, however, I antigen was expressed at levels similar to those in LEA rat livers. The reason for this difference among cell types is still unknown.

The linear structure, i antigen, is mainly expressed in human fetal erythrocytes, and after birth is progressively replaced by the branched structure characteristic of adult erythrocytes.[9] Apparently the expression of i antigen continues in the hepatocytes of adult LEC rats. Very recently, we found that in LEC rat livers expression of a fetal type of hexokinase designated as B type also persists throughout development.[8] However, the expression of α-fetoprotein was not prominent until 15 weeks of age.[29]

In the present study we examined the β1-6GnT activity of 8- to 10-day fetal liver in both LEA and LEC rats (see Table II). In fetal liver of LEA rats at 8–10 days the β1-6GnT activity was found to have already reached 70 pmol/h/mg proteins. These facts indicate that a switch-on of I antigen biosynthesis may occur at an earlier stage of embryogenesis in LEA rats. On the other hand, the fetal liver of LEC rats also contained β1-6GnT activity. After birth, however, the livers of LEC rats almost lack the β1-6GnT activity. These observations indicate that LEC rats lack a switch from i-antigen to I-antigen due to a deficiency of β1-6GnT activity.

The mechanism by which the switch from i- to I-antigen synthesis is prevented in adult LEC rat hepatocytes remains unclear. Numerous observations suggest, however, that conversion from i to I antigen may occur not only during ontogenesis but also during differentiation.[30-32] In order to determine whether the deficiency of I antigen biosynthesis observed in the livers of LEC rats

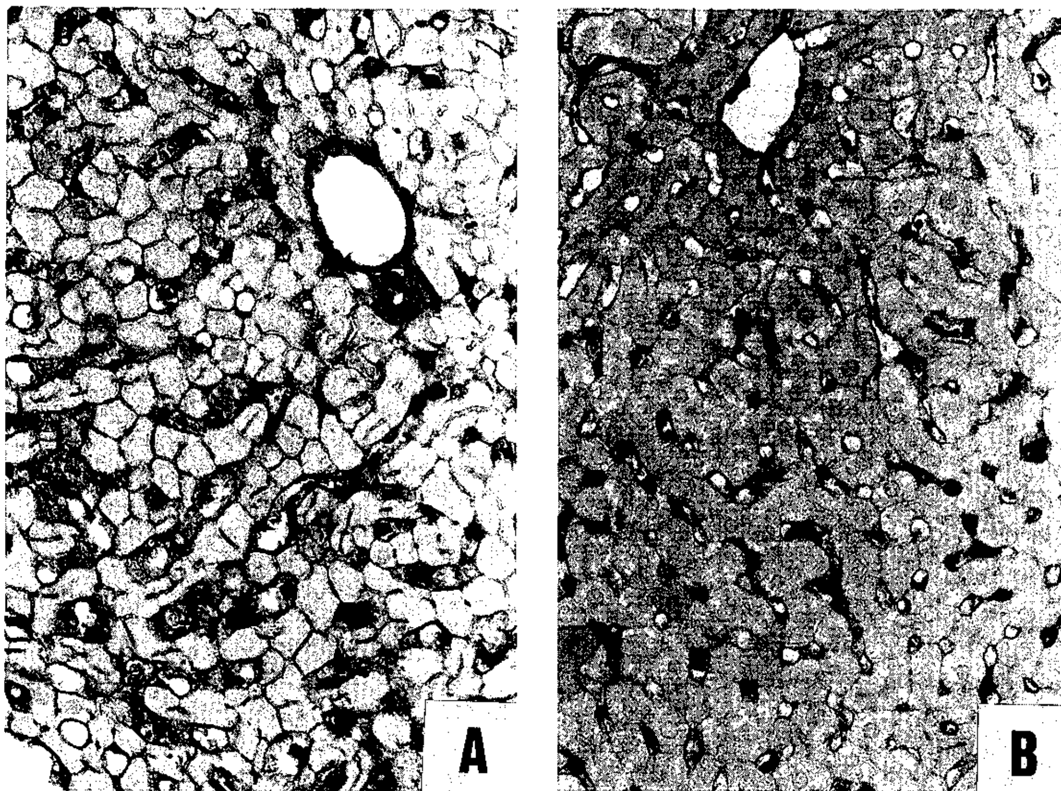

Fig. 2. Immunochemical staining of LEC and LEA rat livers. A, LEA rat liver. B, LEC rat liver.

in this study is a general phenomenon of hepatomas, the β1-6GnT activities of various hepatomas were examined. Rat ascites hepatomas AH-66 and AH-130 and azo-dye-induced hepatoma contained substantial amounts of β1-6GnT, ranging from 88 to 225 pmol/h/mg protein. However, no β1-6GnT activity was found in Yoshida sarcoma, which is a most malignant and highly undifferentiated tumor.[33] This indicates that the absence of I antigen is not a general phenomenon of hepatoma tissues but appears to be a characteristic of the LEC rat liver and highly undifferentiated tumors. At present, however, we do not have any direct evidence that the deficiency of β1-6GnT in the LEC rats is associated with the onset of hepatitis and hepatoma.

Li *et al.* reported that LEC rats have increased levels of copper and metallothionein in the liver and proposed that LEC rats are a model for Wilson's disease.[5] β1-6GnT is inhibited by copper and other metals. However, inclusion of 1 m*M* copper in the assay mixture did not affect the enzyme activity. The decreased activity of β1-6GnT in the LEC rat liver after birth is therefore not related to the high concentration of copper in the tissues. In fact, β1-6GnT activity toward $nLcOse_4$-PA is almost normal in the kidney of LEC rats, where the accumulation of copper has also been reported.[5] Nonetheless, the copper ion could play a role in regulating the gene expression of β1-6GnT after birth, because the accumulation of copper ion has been found in LEC rats aged 4 weeks.[5]

Agui *et al.* reported that the maturation from $CD4^+8^+$ to $CD4^+8^-$ thymocytes was blocked in the T-cells of LEC rats.[4] Whether or not these phenomena are linked with our present observation is a subject for future study.

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