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Abstract

The serum levels of the carbohydrate antigen sialyl Lewis X (SLEX) increase in liver diseases (Sunayama T, Okada Y, Tsuji T., J Hepatol 1994; 19: 451-458). However, it is not known whether the increased serum SLEX levels are associated with the increased levels of its carrier molecules and/or the increased density of SLEX per carrier molecule. By using of rabbit antibody against an SLEX-positive fraction from HepG2 culture supernatant, we developed an enzyme-linked immunosorbent assay to determine the serum levels of the carrier molecules of SLEX (CM_{SLEX}). The CM_{SLEX} -levels in patients with hepatocellular carcinoma were significantly higher than those of normal controls ($P < 0.001$) and benign chronic liver diseases, i.e., chronic active hepatitis, mild and severe form, and liver cirrhosis ($P < 0.05$). Patients with chronic persistent hepatitis and chronic active hepatitis, mild form, had higher CM_{SLEX} -levels than normal controls ($P < 0.05$). The serum CM_{SLEX} -levels did not differ significantly among benign liver diseases. We concluded that serum CM_{SLEX} -levels increase nonspecifically in liver diseases. This is a possible molecular mechanism for the increased serum SLEX levels in liver diseases.

KEYWORDS: glycoprotein, carbohydrate antigen, chronic liver disease, hepatocellular carcinoma

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Increased Serum Levels of the Carrier Molecules of the Carbohydrate Antigen Sialyl Lewis X in Liver Diseases

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The serum levels of the carbohydrate antigen sialyl Lewis X (SLEX) increase in liver diseases (Sunayama T, Okada Y, Tsuji T., *J Hepatol* 1994; 19: 451-458). However, it is not known whether the increased serum SLEX levels are associated with the increased levels of its carrier molecules and/or the increased density of SLEX per carrier molecule. By using of rabbit antibody against an SLEX-positive fraction from HepG2 culture supernatant, we developed an enzyme-linked immunosorbent assay to determine the serum levels of the carrier molecules of SLEX (CM_{SLEX}). The CM_{SLEX}-levels in patients with hepatocellular carcinoma were significantly higher than those of normal controls ($P < 0.001$) and benign chronic liver diseases, *i.e.*, chronic active hepatitis, mild and severe form, and liver cirrhosis ($P < 0.05$). Patients with chronic persistent hepatitis and chronic active hepatitis, mild form, had higher CM_{SLEX}-levels than normal controls ($P < 0.05$). The serum CM_{SLEX}-levels did not differ significantly among benign liver diseases. We concluded that serum CM_{SLEX}-levels increase nonspecifically in liver diseases. This is a possible molecular mechanism for the increased serum SLEX levels in liver diseases.

Key words: glycoprotein, carbohydrate antigen, chronic liver disease, hepatocellular carcinoma

Malignant transformation of cells is often associated with characteristic changes in cellular complex carbohydrates (1, 2). Determination of the serum levels of some carbohydrate antigens has been

used for cancer diagnosis (3, 4). However, phenotypic changes in cellular carbohydrate antigens are not necessarily restricted to cancer cells. Gastric epithelial Tn and T antigen, for example, are not sialylated normally but heavily sialylated in gastric intestinal metaplasias (5). Parenchymal liver cells in chronic liver diseases neoexpress sialyl Lewis X (SLEX) (6, 7). Although hepatocellular carcinomas (HCC) also express the same antigen, the mechanisms for its induction appear to be different between benign and malignant liver diseases (8).

As one glycoprotein carries more than one sugar chain and the same carbohydrate antigen is carried by several different glycoproteins, the expression levels of carbohydrate antigens can be regulated dually, *i.e.*, at the transcription and/or translation level of core peptides and at the post-translational glycosylation level. We previously reported that sera from HCC patients contained SLEX, which was carried almost exclusively by glycoproteins (6), and that the serum levels of SLEX carried by three glycoproteins were elevated in various liver diseases (9). However, we do not know whether increased serum SLEX levels are associated with the increased amount of the carrier molecules of SLEX (CM_{SLEX}) and/or the increased number of SLEX per carrier molecule.

In the present study, we produced rabbit antibody against a high molecular weight SLEX-positive fraction (HMW-SLEX) obtained from a culture supernatant of HepG2, a human HCC cell line secreting SLEX (Okada, unpublished data). With the use of this antibody, we developed an enzyme-linked immunosorbent assay (ELISA) to determine the serum levels of the CM_{SLEX} in various liver diseases. They

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elevated significantly but nonspecifically similar to serum SLEX levels in chronic liver diseases. The highest levels were observed in HCCs. It is suggested that increased serum levels of CM_{SLEX} is partially responsible for the increase in the serum SLEX levels.

Materials and Methods

Preparation of HMW-SLEX. HepG2 cells were grown to be confluent in Dulbecco's modified essential medium (DMEM), supplemented with 10 % fetal calf serum. They were further cultured for 7 days in fresh DMEM without fetal calf serum. The supernatant was concentrated about 50 times in a Minitan Ultrafiltration System with a PLHK cellulose filter membrane (100,000 dalton cut-off) (Japan Millipore Ltd., Tokyo, Japan). Twenty ml of the concentrated supernatant were put into a 5×60 cm Sepharose CL-4B column (Pharmacia-LKB Biotechnology, Uppsala, Sweden) and eluted with 10 mM Tris-HCl, pH 8.0. Fractions of 5 ml were collected and assayed for protein with Lowry's method (10) and SLEX with a radioimmunoassay as described previously (9). A high molecular weight SLEX-positive peak was collected as HMW-SLEX. About 4.5 mg HMW-SLEX as protein were obtained from 1 liter of the supernatant. HMW-SLEX was eluted from a Superose 6-HR 10/30 column (Pharmacia-LKB Biotechnology) at the same position of blue dextran having the molecular weight of 200×10^4 daltons (Sigma Chem. Co., St. Louis, MO, USA).

Immunization and antibody purification.

A female albino rabbit was immunized with 450 μ g of HMW-SLEX emulsified in the complete Freund's adjuvant (Difco Lab., Detroit, MI, USA). Repeated injections of the same amount of the antigen in the incomplete Freund's adjuvant (Difco Lab.) were given three times every 6 weeks. Immune serum referred to as RaAb-SO1 was obtained 10 days after the last injection.

The IgG fraction was purified using a protein A column (Ampure PA kit, Amersham Japan, Tokyo, Japan). The purified antibody was biotinylated with Biotin (long arm)-NHS-water soluble (Vector Lab., Burlingame, CA, USA) according to the manufacturer's instructions.

Antigen specificity of RaAb-SO1. Four

hundred nanograms of HMW-SLEX in 30 μ l of phosphate buffered saline were applied to each well of a 96-well immunoplate (Dynatech Lab. Inc., Chantilly, VA, USA) and incubated overnight at 4 °C for antigen immobilization. After blocking the nonspecific sites with 2.5 % bovine serum albumin (BSA, Cohn fraction V, RIA grade) (Sigma Chem. Co.), 30 μ l of the appropriately diluted RaAb-SO1 was added and incubated at room temperature for 1 h. Eight micrograms of goat anti-rabbit immunoglobulin (Kirkegaard & Perry Lab. Inc., Gaithersburg, MD, USA) radioiodinated at 6.25×10^6 cpm/mg using a chloramine-T method (11) were added and incubated for 1 h. The wells were excised after thoroughly washing and counted in a gamma counter. The specific percentage of binding was about 15 % of the applied radioactivities with 100-times diluted RaAb-SO1. Binding of SH3, a monoclonal antibody (MoAb) against SLEX, to HMW-SLEX was determined as described previously (9).

In an inhibition assay, the binding of RaAb-SO1 to HMW-SLEX was inhibited with 4 to 1000 μ g/ml of MoAb SH3. In another experiment, SH3-binding was inhibited with RaAb-SO1.

The immobilized HMW-SLEX was digested with neuraminidase (1 unit/ml) from *Vibrio cholerae* (Behringwerke, Marburg, Germany) in 50 mM acetate buffer, pH 5.5 at room temperature, overnight, for desialylation. It was also treated with 0.1 M periodate (Sigma Chem. Co.) in 0.1 M acetate buffer, pH 5.6 at 4 °C for 2 h in the dark to abolish all carbohydrate antigenicity. The peptide epitope was denatured by boiling the antigen for 1 h before immobilization.

ELISA for the RaAb-SO1-reactive serum antigen. Blood samples were obtained from each of 10 patients with chronic persistent hepatitis (CPH), chronic active hepatitis, mild form (mCAH), chronic active hepatitis, severe form (sCAH), liver cirrhosis (LC), and HCC, all of whom were hospitalized in the First Department of Internal Medicine, Okayama University Medical School, Okayama, Japan. Diagnoses of chronic hepatitis (CH) and LC were confirmed histologically in all cases. HCCs were confirmed with the use of various imaging techniques. The main tumor size of HCC was more than 3 cm in diameter in all cases. Control sera were obtained from 10 healthy volunteers. Sera was kept frozen at -30 °C until use. Table 1 shows the patients' clinical data.

Table 1 Patient's clinical data

Diagnosis	No.	Age	Sex	AST	γ -GTP	Positive cases for	
		(Year)	M/F	IU/L	IU/L	HBsAg No.(%)	HVcAb No.(%)
Normal	10	34 \pm 5	9/1	nd ^a	nd ^a	0(0)	0(0)
CPH	10	45 \pm 15	10/ 0	41.8 \pm 15.4	42.3 \pm 61.0	1(10)	9(90)
mCAH	10	52 \pm 10	5/5	74.6 \pm 55.9	33.1 \pm 24.1	0(0)	10(100)
sCAH	10	54 \pm 10	8/2	102.9 \pm 63.0	40.8 \pm 26.8	2(20)	8(80)
LC	10	52 \pm 9	9/1	67.3 \pm 48.4	54.3 \pm 36.9	3(30)	7(70)
HCC	10	60 \pm 11	8/2	62.0 \pm 31.7	76.6 \pm 41.1	1(10)	9(90)

^anot determined.

CPH: chronic persistent hepatitis; mCAH and sCAH: chronic active hepatitis, mild form and severe form; LC: liver cirrhosis; HCC: hepatocellular carcinoma.

Fifty microliters of HMW-SLEX at 13.5 μ g/ml were added to the wells of a 96-well immunoplate and incubated at 4°C overnight. After the nonspecific sites were blocked with 2% of BSA and normal rabbit serum, 50 μ l of the reaction mixture containing 2 μ g of biotinylated RaAb-SO1, 1% normal rabbit serum, and 1000-times diluted patients sera were applied and reacted at 4°C overnight. The wells were then washed and 50 μ l of the avidin-biotin-peroxidase complexes was added (Vector Lab.). After incubation at room temperature for 1 h and a thorough washing, 100 μ l of the substrate solution (containing 70 μ g 1, 2-phenylenediamine and 0.01% H₂O₂ in 0.1 M citrate buffer, pH 5.0) was added to each well. After incubation for 8 min at room temperature, the reaction was terminated by the addition of 50 μ l of 2N HCl. Absorbance at 492 nm was read in an ELISA reader. The standard curve was generated in each assay using the serially diluted HMW-SLEX in place of serum antigen. The antigen contained in 675 ng of HMW-SLEX was arbitrarily defined to be one unit.

Sera were digested with 1.25 units/ml of neuraminidase (Behringwerke) at 37°C for 2 h for the assay of CM_{SLEX}. The neuraminidase-treated HMW-SLEX was used to generate the standard curve.

Statistical methods. Results were expressed as arithmetic means with standard deviation (SD). Statistical differences were calculated using a Fisher's protected least significant difference test, and correlations were calculated with a Pearson's correlation coefficient.

Results

Antigen specificity of RaAb-SO1. Periodate treatment of HMW-SLEX abolished almost all SH3-reactivity, as expected (Table 2). The same treatment induced a 30% reduction in RaAb-SO1-reactivity. Essentially the same effects on SH3- and RaAb-SO1-reactivities were obtained with the neuraminidase treatment of HMW-SLEX (Table 2). In contrast, heat treatment of HMW-SLEX reduced the

Table 2 Effects of the treatment of HMW-SLEX with periodate, neuraminidase, and heat on its RaAb-SO1- and SH3-reactivities^a

Treatment	% Specific binding (% inhibition)	
	SO-RI	SH3
(-)	16.2	9.5
Periodate ^b	11.3 (30)	0.1 (99)
Neuraminidase ^c	11.3 (30)	0.4 (96)
Heat ^d	3.3 (80)	8.6 (10)

^aPurified high molecular weight sialyl Lewis X-positive fraction (HMW-SLEX) was placed onto a 96-well immunoplate and reacted with antibody RaAb-SO1 or SH3 as described in Materials and Methods. The bound antibody was detected with ¹²⁵I-labeled anti-rabbit immunoglobulin or anti-mouse IgM. Percentage of specific binding is 100 \times (Bound cpm to the antigen-coated well - Bound cpm to no-antigen well) / Total cpm applied to the well. Percentage of inhibition is 100 \times (% binding without treatment - % binding with treatment) / % binding without treatment.

^bThe antigen was treated with 100 mM periodic acid at 4°C for 2 h.

^cThe antigen was treated with 1 unit/ml of neuraminidase at room temperature overnight.

^dHMW-SLEX was denatured for 1 h in the boiling water bath.

RaAb-SO1-reactivity by 80 % and the SH3-reactivity by 10 %.

Binding of RaAb-SO1 to HMW-SLEX was inhibited with MoAb SH3. The percentage inhibition in-

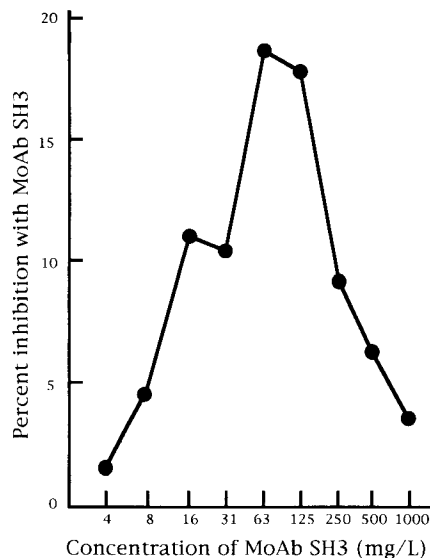


Fig. 1 Inhibitory effect of MoAb SH3 on RaAb-SO1-reactivity. RaAb-SO1-binding to a high molecular weight sialyl Lewis X-positive fraction (HMW-SLEX) was determined in the presence of MoAb SH3 as described Materials and Methods.

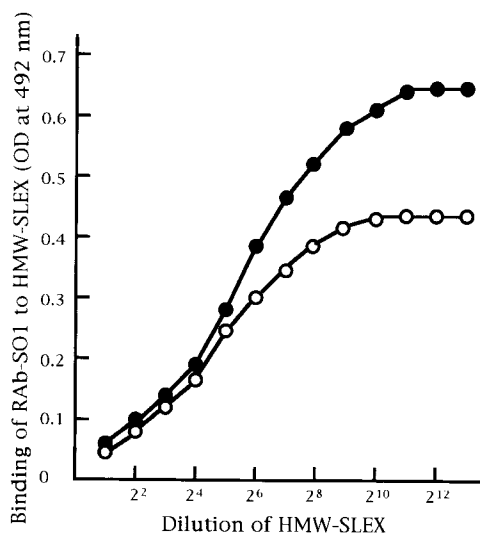


Fig. 2 Effect of desialylation of HMW-SLEX on its RaAb-SO1-reactivity. Binding of RaAb-SO1 to native HMW-SLEX (○—○) and neuraminidase-treated HMW-SLEX (●—●) was inhibited by the serially diluted HMW-SLEX as described in Materials and Methods. HMW-SLEX: See Fig. 1.

Table 3 Serum levels CM_{SLEX} in liver diseases[†]

Diagnosis	No.	Serum CM _{SLEX} levels (Arbitrary units, Mean ± SD)
Normal	10	5.0 ± 1.0
CPH	10	8.4 ± 2.5 ^a
mCAH	10	7.9 ± 3.4 ^d
sCAH	10	7.6 ± 2.9
LC	10	7.5 ± 2.5
HCC	10	11.0 ± 4.2 ^{b,c,d,e}

[†] Sera were desialylated before the assay as described in Materials and Methods.

CM_{SLEX}: carrier molecule of sialyl Lewis X; CPH: chronic persistent hepatitis; mCAH and sCAH: chronic active hepatitis, mild form and severe form; LC: liver cirrhosis; HCC: hepatocellular carcinoma. ^a*P* < 0.05 compared with Normal. ^b*P* < 0.01 compared with normal. ^c*P* < 0.05 compared with mCAH. ^d*P* < 0.05 compared with sCAH. ^e*P* < 0.05 compared with LC.

creased as the SH3 concentration in the reaction mixture was increased and reached a maximum inhibition rate of 18 % at 64 to 128 μg/ml of SH3 (Fig. 1). Inhibition decreased at higher SH3 concentrations. SH3 binding to HMW-SLEX was inhibited almost completely with RaAb-SO1.

Altogether, the above results suggest that 70 to 80 % of RaAb-SO1-reactivity is directed against the carrier molecules of SLEX, *i.e.*, CM_{SLEX} and 20 to 30 % against the sialylated carbohydrate antigens. RaAb-SO1-reactivity against carbohydrate antigens was eliminated with the neuraminidase treatment of the antigens as effectively as the periodate treatment.

Serum levels of CM_{SLEX} in various liver diseases. The serum levels of CM_{SLEX} were determined using the neuraminidase-treated sera.

The HCC patients had the highest serum levels of CM_{SLEX} (Table 3). They were significantly higher than those of the normal controls (*P* < 0.01), mCAH (*P* < 0.05), sCAH (*P* < 0.05) and LC (*P* < 0.05). Patients with CPH and mCAH also had the significantly higher levels than the normal controls (*P* < 0.05). No significant difference was observed among the benign liver diseases. The proportion of patients with abnormally high CM_{SLEX} levels were similar among CPH, mCAH, sCAH, LC and HCC (Fig. 3).

The standard curve obtained using the neuraminidase-treated HMW-SLEX was depressed about 30 % in comparison with the curve generated using the native antigen (Fig. 2). In contrast to HMW-

SLEX, the neuraminidase treatment apparently increased the RaAb-SO1-reactivities of the serum antigens (Table 4). They increased about $4 \times$ in the

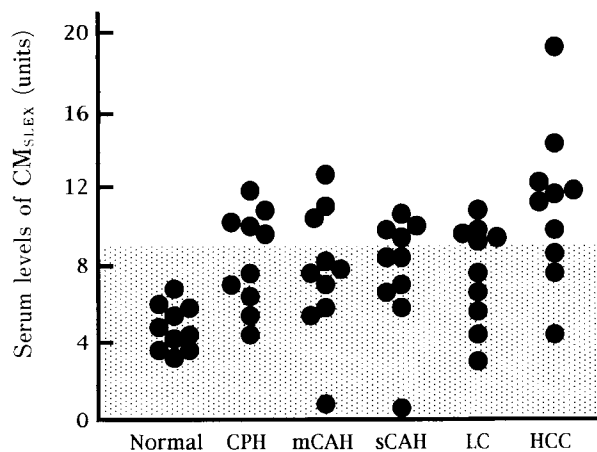


Fig. 3 Serum levels of the carrier molecules of sialyl Lewis X (CM_{SLEX}) in various liver diseases. Dotted area shows the normal range, *i.e.*, less than the mean $+ 4 \times$ SD of the normal controls. CPH: chronic persistent hepatitis; mCAH: chronic active hepatitis, mild form; sCAH: chronic active hepatitis, severe form; LC: liver cirrhosis; HCC: hepatocellular carcinoma.

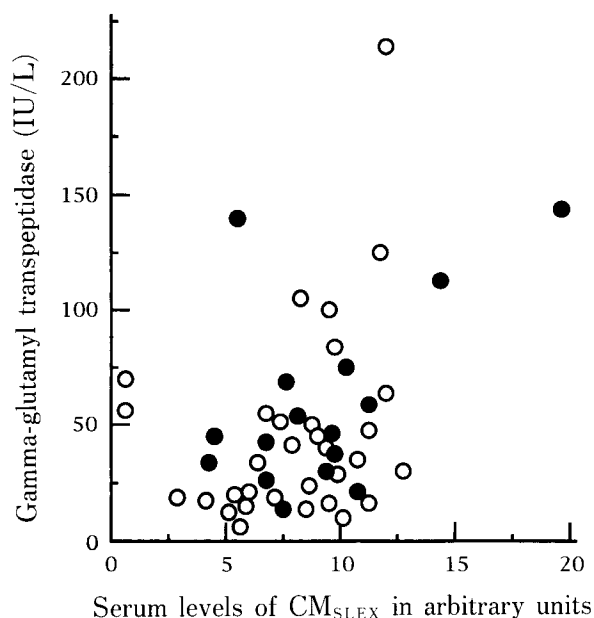


Fig. 4 Correlation between the serum levels of CM_{SLEX} and γ -GTP. Open and closed circles denote nonalcoholics and alcoholics, respectively. CM_{SLEX} : See Fig. 3.

Table 4 Changes in the RaAb-SO1-reactivity before and after desialylation of the serum antigens and HMW-SLEX

Antigens	Ratio of RaAb-SO1-reactivity after to before desialylation ^a
Sera from	
Normal	4.2
CPH	4.4
mCAH	4.2
sCAH	2.2
LA	2.3
HCC	2.8
HMW-SLEX	0.7

^aSpecific % RaAb-SO1 binding after desialylation was divided by that before desialylation.

CPH: chronic persistent hepatitis; mCAH and sCAH: chronic active hepatitis, mild form and severe form; LC: liver cirrhosis; HCC: hepatocellular carcinoma. HMW-SLEX: See Table 2.

normal controls, CPH and mCAH, and about 2 to $3 \times$ in sCAH, LC and HCC. The correlation between the serum levels of SLEX and CM_{SLEX} ($r = 0.151$, $P = 0.25$) was not significant.

Serum levels of CM_{SLEX} showed a significant correlation with those of γ -glutamyl transpeptidase ($r = 0.359$, $P < 0.05$) (Fig. 4). However, there was no significant difference in the CM_{SLEX} levels between the patients with and without a history of alcoholism, *i.e.*, more than 540 ml-Japanese sake per day for more than 10 years 9.3 ± 1.0 vs. 8.1 ± 0.5 , $P = 0.224$. No significant correlation was found between the peripheral levels of CM_{SLEX} and those of alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, albumin, γ -globulin, alkaline phosphatase, total cholesterol, and plasma disappearance rate of indocyanine-green. Age, gender, and the positivity of hepatitis B surface antigen and hepatitis C virus antibody showed no correlation with CM_{SLEX} levels.

Discussion

SLEX is a carbohydrate antigen reported to be specifically expressed by malignantly transformed cells (12, 13). However, we have previously reported that parenchymal liver cells in both inflammatory and malignant lesions neoexpressed SLEX (6-8). Plasma SLEX levels also increased in these disorders (9). In

the present study, we looked at whether increased serum SLEX levels were associated with the increase in its carrier molecules. Changes in both serum levels and lectin reactivity, *i.e.*, the structure of complex carbohydrate, of α -fetoprotein are observed in HCC patients (14, 15).

We used rabbit antibody against the SLEX-positive antigens, *i.e.*, HMW-SLEX, from the culture supernatant of HepG2. Both the periodate- and neuraminidase-treatment of HMW-SLEX decreased its antibody-reactivity by about 30 %. Heat denaturation, on the other hand, decreased it by 80 %. In our previous studies, we have shown that most serum SLEX is carried by glycoproteins (6, 9). Altogether, these results suggest that 70 to 80 % of antibody-reactivity was directed against the peptide antigens, and 20 to 30 % against the sialylated carbohydrate antigens.

Patients with liver disease had higher serum levels of CM_{SLEX} than normal controls. They elevated non-specifically with the serum SLEX levels (9). The levels in benign liver disease patients and HCC patients, respectively, were about 1.5 and 2.2 times higher than the normal values. The SLEX levels in these patients were about 4 times higher than the normal values (9). These results suggest that not only the increased serum levels of CM_{SLEX} but also the increased SLEX-density-per-carrier-molecule were responsible for the observed increase in serum SLEX levels.

Serum SLEX levels did not differ significantly between benign and malignant liver diseases (9). However, serum CM_{SLEX} levels were significantly higher in HCC patients than in benign liver disease patients. Serum SLEX was carried by several different glycoproteins (6, 9). We previously suggested that the induction mechanism of SLEX might differ between benign and malignant liver diseases (8). It will be interesting to study whether a particular SLEX-positive glycoprotein is increased specifically in HCC patients.

Neuraminidase treatment decreased RaAb-SO1-reactivity against HMW-SLEX but increased its reactivity against the serum antigen 2 to 4 \times . This unexpected result suggests differences between HMW-SLEX and the serum antigens in their glycoprotein composition and/or glycosylation rate. Desialylation of serum antigens might induce in them

conformational changes, which uncovers the cryptic peptide epitopes for RaAb-SO1. The resulting increase in RaAb-SO1-reactivity against CM_{SLEX} might be much greater than the decrease in the reactivity against the carbohydrate antigens. On the other hand, desialylation would not affect RaAb-SO1-reactivity against peptide epitopes of HMW-SLEX, and might simply decrease the anti-carbohydrate antigen-reactivity. It is interesting that the ratio of serum RaAb-SO1-reactivity after to before desialylation was higher in the less severe liver diseases. This may indicate differences in glycosylation rate among various liver diseases. Glycoproteins synthesized by cancer cells were shown to be less glycosylated (16, 17).

There was a significant correlation between serum CM_{SLEX} and γ -GTP levels. Higher serum γ -GTP levels are often observed in alcoholics. However, probably because we examined the patients during hospitalization in the present study, the serum γ -GTP levels of the alcoholics and nonalcoholics were similar. Furthermore, the serum CM_{SLEX} levels did not differ significantly between alcoholics and nonalcoholics. These observations indicate that the serum CM_{SLEX} levels were not directly related to alcoholic history.

The pathobiological function of SLEX and increased levels of serum SLEX is unknown. SLEX is a ligand for the endothelial leukocyte adhesion molecule 1 (18, 19). Neoexpression of SLEX on HCC cell surfaces may promote HCC cell adhesion to endothelial cells in metastasis. Increased SLEX expression by colorectal cancer has been found to be associated with higher metastatic potential (20). On the other hand, increased serum SLEX may competitively inhibit the adhesion of neutrophils to endothelial cells, which may be related to a higher frequency of bacterial infection in chronic liver diseases.

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