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Detailed Characterization of a High-molecular-weight Glycoprotein Secreted by Lung Cancer Cells

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A cancer-associated, high-molecular-weight glycoprotein antigen (6B3·Ag) recognized by monoclonal antibody 6B3 was purified from culture medium of human large cell lung carcinoma cell line (HLC-2) and characterized biochemically and immunochemically. The 6B3·Ag was purified more than 1,200-fold with a yield of 30% by salting out, precipitation by acidification at pH 4.5, and chromatographies on Sepharose 4B and concanavalin A-Sepharose. The molecular weight of 6B3·Ag is approximately 1,000,000 and the molecule is a homodecamer of 94,000 subunits. The 6B3·Ag is a glycoprotein containing 22.9% sugars, consisting of both *N*- and *O*-glycoside chains. The N-terminal 19 amino acids were determined and only 4 out of 19 amino acid residues were different from those of an antigen, L3, secreted by lung carcinoma cell line Calu-1. The serum level of 6B3·Ag was determined in normal adults as well as patients with various diseases by enzyme-linked immunosorbent assay. The mean serum level of 6B3·Ag was 3.1 μg/ml, ranging from 1.6 to 6.2 μg/ml in 131 healthy adults. When the cut-off value was set at 6.2 μg/ml, the incidence of positive values in the sera was elevated not only in malignant diseases such as hepatoma (73%) and leukemia (62%), but also in benign diseases such as chronic hepatitis (42%) and liver cirrhosis (63%). While the incidence of positive values was elevated in advanced liver diseases, namely, chronic hepatitis, liver cirrhosis and hepatoma, the cancer specificity of 6B3·Ag did not appear to be high.

Key words: High-molecular-weight glycoprotein — Chemical properties — Serum level

Several investigators have searched for antigens specific for lung cancer by using monoclonal antibody techniques.[1–7] Monoclonal antibodies (mAbs) against a human large cell lung carcinoma cell line[8] (HLC-2) have been prepared, and their antigens examined. Among them, a mAb 6B3 showed little immunohistochemical reaction to normal tissues, but various adenocarcinoma tissues were stained well by the mAb. It was found that an antigen (6B3·Ag) recognized by the mAb was secreted in a large amount from the HLC-2 cell line, and was present in the serum of cancer patients at a high level. The 6B3·Ag was separated from HLC-2 culture medium in order to investigate the possibility that this antigen might be useful as a tumor marker, and its biochemical and immunological characteristics were examined. Furthermore, an enzyme-linked immunosorbent assay (ELISA) system for the determination of 6B3·Ag was established and the serum level of the antigen was determined in patients with various diseases.

During the progress of this study, it was clarified by the analysis of N-terminal amino acid sequences of 6B3·Ag that the antigen possesses characteristics quite similar to those of an antigen, L3, studied by Linsley *et al.*,[9] for which only the N-terminal amino acid sequence, molecular weight, subunit structure, sugar components and contents, and the serum level in patients, etc. have so far been reported. L3 antigen was also identical to melanoma-associated antigen reported by Natali *et al.*[10] The correlation between serum level and disease for melanoma-associated antigen has not been clarified.

MATERIALS AND METHODS

Reagents α-Methyl-D-mannoside, bovine serum albumin (BSA, type V) and tunicamycin were purchased from Sigma Chemical Co. (St. Louis, MO), Coomassie Brilliant Blue R-250 from Nakarai Chemical, Ltd. (Kyoto), RPMI-1640 medium and fetal calf serum (FCS) from Gibco Ltd. (Grand Island, NJ), tissue culture flasks (750 ml, No. 3028) from Falcon Plastics (Cockeysville, MD), Sepharose 4B, concanavalin A (Con A)-Sepharose 4B and molecular weight standards for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) from Pharmacia Chemicals AB (Uppsala, Sweden), 96-well ELISA plates (Immunoplate II) from Nunc (Wiesbaden, Germany), alkaline phosphatase (ALP, EIA grade) from Boehringer Mannheim Co. (Mannheim, Germany), gelatin from Difco Laboratories (Detroit, MI), and penicillin and streptomycin from Meiji Seika Kaisha, Ltd. (Osaka).

Materials HLC-2 cells established in our laboratories[8] were maintained in monolayer culture in RPMI-1640 medium containing 50 units/ml of penicillin, 50 μg/ml of streptomycin and 5% FCS. The cells (2×10^7) were cultured in a 750 ml tissue culture flask (No. 3028) for 6 days at 37°C in a 5% CO_2-95% humidified atmosphere. The culture medium was harvested and preserved at −20°C until used for purification of 6B3·Ag.

Methods of preparation of mAbs including 6B3 mAb to HLC-2[8] were as follows: hybridomas secreting anti-HLC-2 cell mAbs were derived by fusing the murine myeloma cell line P3-X63-Ag8-U1 (P3U1) with splenocytes from BALB/c mice immunized by two i.p. injections of 1×10^7 HLC-2 cells. The specific mAbs were screened immunohistochemically through panels of non-neoplastic or neoplastic cultured cells. For characterization of its properties, the antigen recognized by the 6B3 mAb was purified further from HLC-2 culture medium by immunoaffinity column chromatography using 6B3 mAb (IgG_1, subclass) as will be described in the next section. The partially purified antigen obtained was used to immunize mice, and new mAbs (#K35A3 and #O-10) were obtained by the standard hybridoma technique.[11]

The sera of normal adults and patients with various diseases were kindly provided by Dr. T. Kohji (Nagasaki University).

Purification of 6B3·Ag from HLC-2 culture medium The 6B3·Ag was partially purified by immunoaffinity column chromatography using 6B3 mAb. The culture medium (2 liters) was centrifuged at 10,000*g* for 10 min, and to the supernatant was added 180 g of sodium sulfate per liter of the culture medium. Precipitates obtained by centrifugation at 5,000*g* for 10 min were dissolved and dialyzed in 10 m*M* phosphate-buffered saline, pH 7.2 (PBS). The dialysates were combined and applied to a 6B3 mAb-coupled Sepharose 4B column (2.5×20 cm; 5 mg of mAb bound per ml of the gel). After standing at 4°C for 18 h, the column was washed extensively with PBS containing 0.5 *M* NaCl. The 6B3·Ag was eluted with 3 *M* NaSCN, and dialyzed against PBS. This fraction was used as partially purified 6B3·Ag.

The purification of 6B3·Ag on a large scale was performed as described below, and the antigenicity of the antigen was measured by ELISA. The culture medium (20 liters) was fractionated by adding 180 g of sodium sulfate per liter to it, and the precipitates obtained were dissolved and dialyzed in distilled water. Acetate buffer (0.5 *M*, pH 4.5) was added to the dialysate at the final concentration of 25 m*M*, resulting in the formation of precipitates, which were collected and dissolved in 10 m*M* Tris-HCl buffer, pH 8.0. The precipitation and successive solubilization procedures with the acetate buffer were repeated twice. The resulting solution was dialyzed against buffer A (0.1 *M* acetate buffer, pH 6.0, containing 0.5 *M* NaCl) and applied to a Sepharose 4B column (2.5×90 cm) pre-equilibrated with the same buffer, followed by elution with the same buffer. Fractions which react with anti-6B3·Ag mAb were collected and applied to a Con A-Sepharose 4B column (2.5×30 cm) pre-equilibrated with buffer A. The column was allowed to stand at 4°C for 18 h, then the proteins bound to the column were eluted with 0.1 *M* α-methyl-D-mannoside. The eluate was then applied to the Sepharose 4B column again, and the 2nd peak reacting with anti-6B3·Ag mAb on ELISA was rechromatographed on the same column.

Zinc turbidity test (ZTT) and type IV collagen in patients' sera A commercial kit for ZTT (Shino-Test Corp., Tokyo) or type IV collagen (Fuji Chemical Industries, Ltd., Toyama) was used. ZTT (a test of hepatic functions) was measured as follows: 100 μl of serum and 6.0 ml of 0.16 m*M* $ZnCl_2$ solution were mixed and incubated for 30 min at 25°C, then the absorbance at 660 nm was measured with a Hitachi 228 spectrophotometer (Hitachi, Tokyo). Type IV collagen, which may be related to liver fibrosis,[12,13] was measured by one-step sandwich enzyme immunoassay (EIA). A 20 μl aliquot of type IV collagen solution or serum was incubated with a polystyrene ball coated with mouse mAb to human type IV collagen and with 300 μl of mouse mAb to human type IV collagen labeled with peroxidase in PBS for 1 h at room temperature. After the incubation, the polystyrene ball was washed 3 times with PBS and then incubated with 300 μl of 3,3′,5,5′-tetramethylbenzidine (0.134 g/liter) and 100 μl of H_2O_2 (0.1 g/liter) for 30 min at room temperature. The reaction was stopped by adding 600 μl of sulfuric acid (0.67 mol/liter), and the absorbance at 450 nm was measured with a Hitachi 228 spectrophotometer.

ELISA for 6B3·Ag All procedures were carried out at 37°C. One hundred μl of mAb (#O-10) to 6B3·Ag in PBS (protein 50 μg/ml) was coupled to each well of a 96-well ELISA plate by incubation for 2 h. The wells were then incubated with 200 μl of 1% gelatin in PBS for 1 h to block non-specific binding before the reaction of the mAb with its specific antigen. One hundred μl of the samples diluted more than tenfold with PBS or 10% goat serum in the case of serum was added to each well, and the plate was incubated for 1 h. After washing twice with PBS, 100 μl of #K35A3 mAb labeled with ALP was added to each well and left for 1 h. After washing of the wells with PBS, 100 μl of substrate solution containing 4 m*M* phenylphosphate and 2 m*M* 4-aminoantipyrine in 20 m*M* carbonate buffer (pH 10.2) was added and incubation was continued for 30 min. The reaction was stopped by adding 100 μl of 20 m*M* $NaIO_4$. The absorbance at 510 nm was measured with a microtiter plate analyzer (NJ-2000, Nippon InterMed K.K., Tokyo).

Table I. Specific Activity of 6B3·Ag during Purification Procedure

Step	Volume (ml)	Total protein (mg)	Total 6B3·Ag (mg)	Specific activity (μg 6B3·Ag/mg protein)	Yield (%)
HLC-2 cul. sup.	20,000	45,000	36.5	0.8	100
18% Na_2SO_4 ppt	280	3,360	32.2	9.5	85
pH 4.5 ppt	50	620	25.0	40.3	68
Sepharose 4B	28	63	20.8	330	57
Con A-Sepharose 4B	15	32	16.5	515	45
Sepharose 4B (1st)	10	16	14.0	875	36
Sepharose 4B (2nd)	10	11	11.0	1,005	30

Chemical analysis of 6B3·Ag Total protein was determined by Lowry's method[14] using BSA as a standard. The 6B3·Ag used as the ELISA standard was prepared on a dry weight basis. Sugar content and composition of 6B3·Ag were analyzed by gas-liquid chromatography with a glass column of 5% SE-30/Chromosorb WAWDMCS on a Hitachi 663-30 gas chromatograph. Sugars were analyzed as their trimethylsilyl derivatives.[15, 16] Mannitol was used as an internal standard. In order to investigate the nature of the saccharide moiety of the 6B3·Ag, HLC-2 cells (2×10^7) were cultured in the above-described culture medium containing 1 μg of tunicamycin[17] for 6 days. For the determination of N-terminal amino acid sequence, automated Edman degradation was performed with approximately 100 pmol of 6B3·Ag in a gas-phase sequencer (Model 473A, Applied Biosystems, Inc., Foster City, CA).

Electrophoresis of 6B3·Ag SDS-PAGE of 6B3·Ag was carried out according to the method of Laemmli.[18] The bands were stained with Coomassie Brilliant Blue R-250. Agarose electrophoresis was carried out in 1.2% agarose gel with 75 m*M* barbital buffer (pH 8.6, $\mu=0.05$), under a constant current of 2 mA/cm.

RESULTS

Purity The results of 6B3·Ag purification are summarized in Table I. The 6B3·Ag was purified more than 1,200-fold from HLC-2 culture medium with a yield of 30% (in terms of antigenicity of 6B3·Ag) by salting out, precipitation by acidification at pH 4.5 and chromatographies on Sepharose 4B and Con A-Sepharose. This preparation gave a single spot in the pre-β globulin region on agarose gel electrophoresis (Fig. 1).

Molecular weight The molecular weight (MW) of native 6B3·Ag was estimated to be 1,000,000 (1,000k) by chromatographic analysis on a Sepharose 4B column (2.5×90 cm) (Fig. 2). The 6B3·Ag from freshly prepared HLC-2 culture medium or human serum was eluted at a position corresponding to approximately 1,000k on a Sepharose 4B column (Fig. 3a, b). On SDS-PAGE (12% gel), a single band corresponding to a MW of 94k was obtained for the purified 6B3·Ag under reducing (Fig. 4A) and non-reducing conditions. The 94k subunit was labile and easily converted into two fragments of 66k and 27k after storage at 4°C for a month (Fig. 4B), but the MW of whole 6B3·Ag observed on gel-filtration was not changed.

Fig. 1. Agarose electrophoresis of 6B3·Ag. a, human serum; b, 6B3·Ag.

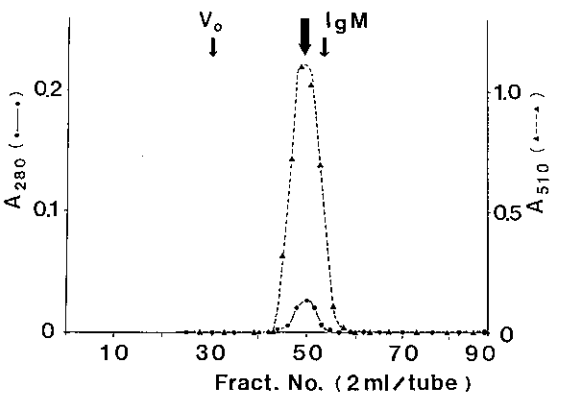

Fig. 2. Elution profile of 6B3·Ag on a Sepharose 4B column. 6B3·Ag (0.3 mg) was applied to a Sepharose 4B column (2.5×90 cm) equilibrated with buffer A. Closed circles indicate absorbance at 280 nm. 6B3·Ag was detected by ELISA (▲). The molecular weight of IgM was 900k. V_o indicates the position of the void volume.

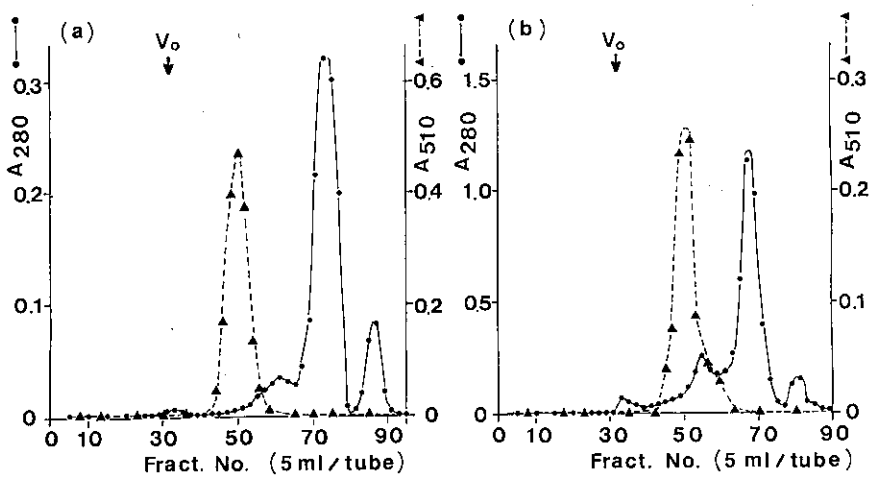

Fig. 3. Elution profile of 6B3·Ag from freshly prepared HLC-2 culture medium or human serum on a Sepharose 4B column. Five ml of HLC-2 culture medium containing 6B3·Ag at 2 μg/ml (a) or 1 ml of human serum containing 6B3·Ag at 3 μg/ml (b) was applied to a Sepharose 4B column (2.5×90 cm) equilibrated with buffer A. Closed circles indicate absorbance at 280 nm. 6B3·Ag was detected by ELISA (▲). V_o indicates the position of the void volume.

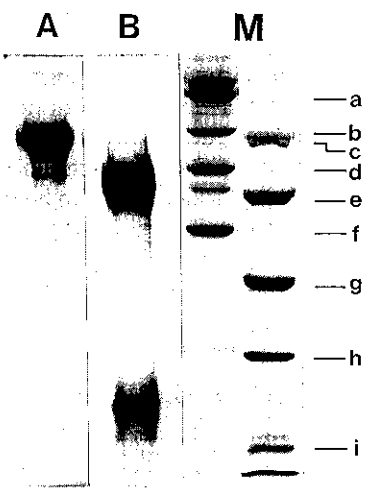

Fig. 4. SDS-PAGE (12% gel) of 6B3·Ag under reducing conditions (2% 2-mercaptoethanol). Marker proteins (M) are as follows: a, bovine plasma α_2-macroglobulin (170k); b, *E. coli* β-galactosidase (116k); c, rabbit muscle phosphorylase *b* (94k); d, human transferrin (76k); e, bovine serum albumin (67k); f, bovine liver glutamic dehydrogenase (53k); g, ovalbumin (43k); h, bovine erythrocyte carbonic anhydrase (30k); and i, soybean trypsin inhibitor (20k). A, fresh 6B3·Ag; B, 6B3·Ag kept at 4°C for one month.

Sugar composition The 6B3·Ag contained 22.9% sugars. Fucose, mannose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and *N*-acetylneuraminic acid were detected by gas-liquid chromatography (Table II). After tunicamycin treatment of HLC-2 cells, the subunit of 6B3·Ag exhibited a smaller MW on SDS-PAGE, indicating the presence of *N*-glycoside chains in 6B3·Ag.

Amino-terminal sequence The N-terminal (19 residues) amino acid sequence of the 6B3·Ag was compared with that of L3 antigen reported by Linsley *et al.*[9] (Table III).

Table II. Analysis of Sugar Composition of 6B3·Ag

Component	μg/mg (6B3·Ag)	
Fucose	11	11.2%
Mannose	45	
Galactose	56	
Glc-NAc	63	8.8%
Gal-NAc	25	
Neu-NAc	29	2.9%
Total	229	22.9%

Sugar contents are given as sugar μg/mg dry weight of 6B3·Ag, obtained by gas-liquid chromatography. Mannitol was used as an internal standard.

Table III. N-Terminal Sequence of 6B3·Ag Compared with L3 Antigen

	N-1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
6B3·Ag	V	N	T	G	Q	M	W	Q	A	D	G	G	A	T	N	Q	G	A	V
L3·Ag	V	N	D	G	D	M	R	L	A	D	G	G	A	T	N	Q	G	A	V

Fifteen amino acids in the N-terminal sequence were identical to those of L3 antigen, but 4 were different.

Serum level of 6B3·Ag A sandwich ELISA for 6B3·Ag in serum was developed. The standard calibration curve is shown in Fig. 5. The range of 30 to 500 ng/ml of 6B3·Ag could be quantitatively determined. The intra-assay coefficients of variation (N=10) were 5.6 to 7.6% for 30 to 480 ng/ml of 6B3·Ag, while the inter-assay coefficients of variation (N=10) were 6.7 to 9.5%. The validity of the assay was confirmed by stepwise dilution of samples and analytical recovery tests.

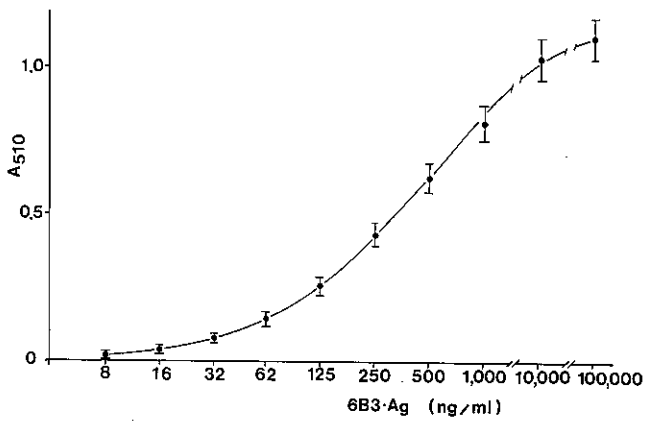

Fig. 5. Standard curve of 6B3·Ag for ELISA. Vertical bars indicate the mean±SD (N=5).

Serum 6B3·Ag levels in 131 healthy adults showed a logarithmic distribution, with a mean ($\bar{x}$) of 3.1 μg/ml, $\bar{x}-2$ SD of 1.6 μg/ml and $\bar{x}+2$ SD of 6.2 μg/ml. When the cut-off value was set at 6.2 μg/ml ($\bar{x}+2$ SD), the incidence of positive values was high in the sera from patients with hepatoma (73%), leukemia (62%) and some other cancers (Fig. 6), as well as in the sera from patients with benign diseases such as chronic hepatitis (42%), liver cirrhosis (63%) and other.

Correlation of the 6B3·Ag with liver diseases The correlation of 6B3·Ag with type IV collagen or ZTT was studied to assess the significance of elevation of serum 6B3·Ag levels. The level of 6B3·Ag in the serum of liver disease patients did not correlate with that of type IV collagen (coefficient of correlation; $r=-0.00063$, N=42), which may be relevant to liver fibrosis[12, 13] (Fig. 7a), while the level correlated slightly with ZTT ($r=0.479$, N=100), a test of hepatic functions (Fig. 7b).

DISCUSSION

In this study, an antigen (6B3·Ag) recognized by a mouse mAb to human large cell lung carcinoma was isolated and partially characterized (molecular weight, subunit composition and sugar composition). Furthermore, the serum levels of this antigen in normal individuals and various cancer patients were determined.

The 6B3·Ag is a glycoprotein of high molecular weight (approximately 1,000k), which seems to be present in the serum as a decamer of identical 94k subunits judging

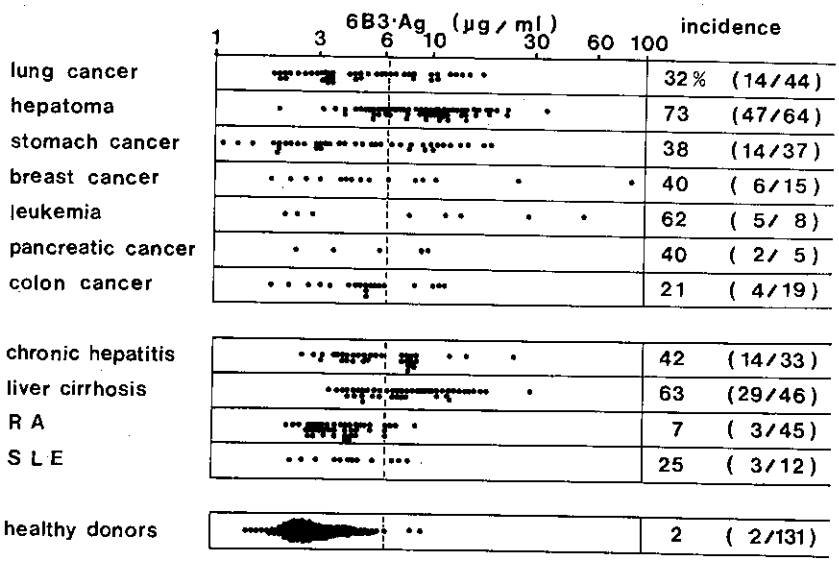

Fig. 6. Levels of 6B3·Ag in sera of healthy adults and patients with various carcinomas or benign diseases. The concentration of 6B3·Ag in human serum was determined by ELISA. The cut-off value was set at 6.2 μg/ml ($\bar{x}+2$ SD), and the incidence of positive values was obtained. RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

from the results of this study. The 94k subunit was labile and easily converted into two fragments of 66k and 27k during storage. We can not rule out the possibility that 6B3·Ag possesses a weak protease activity, or that some proteases are present in purified 6B3·Ag in very small quantities. Linsley *et al.* reported that the L3 antigen had three molecular forms of 94, 76 and 27k, and they supposed the 76k form (cellular form) to be a precursor of the 94k form (released form). Considering the results of the present study and Linsley's report, it seems that the linkage between 66k and 27k components may be weak in the 94k subunit, and this phenomenon may have some special significance. A further study is needed.

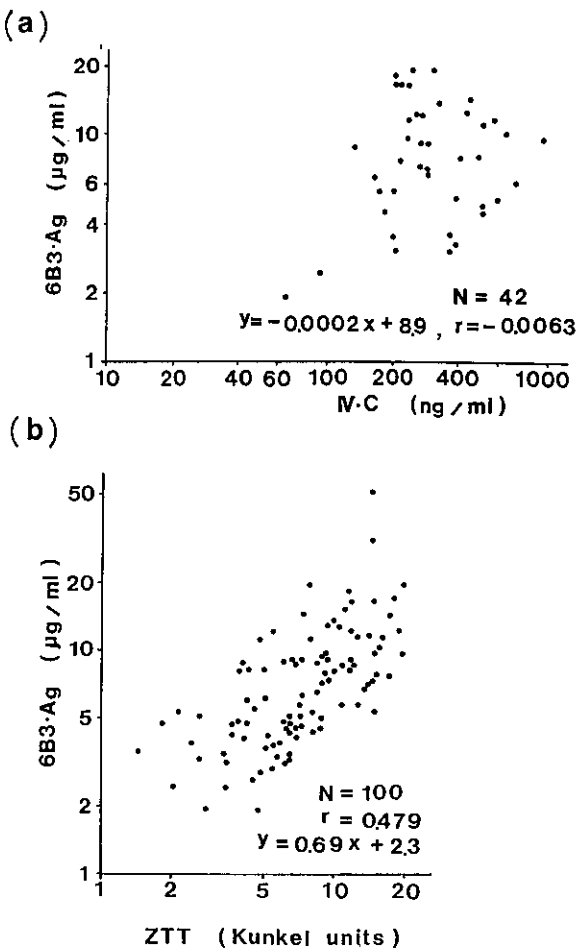

Fig. 7. Correlation between concentration of 6B3·Ag and type IV collagen (IV·C) or ZTT in sera of patients with liver diseases. (a) The concentration of IV·C was measured by one step sandwich EIA. Twenty μl of patient's serum or IV·C standard solution was incubated with a polystyrene ball coated with mAb to human IV·C and with 300 μl of mAb to human IV·C labeled with peroxidase for 1 h at room temperature. After being washed three times with PBS, the polystyrene ball was incubated with 300 μl of 3,3′,5,5′-tetramethylbenzidine (0.134 g/liter) and 100 μl of H_2O_2 (0.1 g/liter) for 30 min at room temperature. The reaction was stopped by adding 600 μl of sulfuric acid (0.67 mol/liter) and the absorbance at 450 nm was measured. (b) The unit of ZTT was measured as follows: One hundred μl of patient's serum and 6.0 ml of 0.16 m*M* $ZnCl_2$ solution were mixed and incubated for 30 min at 25°C, and the absorbance at 660 nm was measured. The concentration of 6B3·Ag in serum was determined by ELISA.

The 6B3·Ag is highly glycosylated protein containing 22.9% sugars, composed of fucose, mannose, galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine and *N*-acetylneuraminic acid. Microheterogeneity of sugar moiety may exist. The cell culture experiment with tunicamycin resulted in a shift of the subunit of 6B3·Ag to smaller MW on SDS-PAGE, and the identification of a high *N*-acetylgalactosamine content (10.9%) in the 6B3·Ag, suggesting the presence of both *N*-linked and *O*-linked sugars in the molecules.

The 6B3·Ag was hardly present in normal human tissues studied, and it appeared to be nonspecific to lung cancer. But the 6B3·Ag was shown to be localized in various adenocarcinoma tissues. Since 6B3·Ag was elevated in serum of some cancer patients, it could be a new tumor marker. However, this point should be carefully examined, because the N-terminal amino acid sequence of 6B3·Ag is identical with that of L3 antigen reported by Linsley *et al.* at 15 out of the first 19 amino acids, so that the relationship between these two antigens will need to be more fully investigated. In this connection, it might be pointed out that L3 was found to be non-specific to lung cancer.

The serum level of 6B3·Ag in 131 healthy adults was determined. Its distribution was logarithmic, and its mean level of 3.1 μg/ml was almost the same as that of L3 antigen. When the cut-off value was set at 6.2 μg/ml, positive incidence was high in patients with hepatoma (73%), leukemia (62%), stomach cancer (38%), breast cancer and pancreatic cancer (40%) and colon cancer (21%) (Fig. 6). The incidence of positivity was 32% in patients with lung cancer. Lung adenocarcinoma and large cell lung carcinoma tissues were immunohistochemically stained well and at low frequency, respectively, by the 6B3 mAb, when serum from accurately diagnosed lung cancer patients was used, and the incidence of positivity appeared to vary widely among the four different histological types of lung carcinoma. On the other hand, the incidence of positivity was high in benign diseases such as liver diseases, especially in liver cirrhosis (63%). The serum level of 6B3·Ag in patients with liver diseases did not correlate with that of type IV collagen, but correlated weakly with ZTT (Fig. 7a, b). The results suggested that 6B3·Ag does not provide information on liver fibrosis but is related to a morbid state reflected by ZTT, for example, the quantity of

serum immunoglobulins. In a preliminary study, it was found that 6B3·Ag was partly present as a complex with IgG and/or IgM in human serum, and purified 6B3·Ag showed affinity for immunoglobulins. These are interesting results, considering the general decline of immune function in neoplastic disease. Clarification of the biological activity of 6B3·Ag should give us a better idea of its potential value as a diagnostic marker.

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