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Ganglioside antigen of DU-PAN-2 in a human pancreatic cancer

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Abstract DU-PAN-2 reactive gangliosides were isolated from the tumor of a patient with pancreatic cancer (duct cell carcinoma, moderately differentiated adenocarcinoma), having a negative Lewis blood phenotype, and were analyzed by means of TLC-immunostaining, enzyme-linked immunosorbent assay (ELISA), permethylation study, ^{1}H NMR spectroscopy and fast atom bombardment mass spectrometry. The structures of the gangliosides were found to be NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-1'Cer, containing normal and hydroxy fatty acids. By TLC-immunostaining and ELISA with chemically synthesized gangliosides, DU-PAN-2 was demonstrated to react strongly with IV^{3}αNeuAc-Lc$_4$Cer, weakly with IV^{3}αNeuAc-nLc$_4$Cer, and moderately with IV^{6}αNeuAc-Lc$_4$Cer and IV^{6}αNeuAc-nLc$_4$Cer. Thus it was concluded that the DU-PAN-2 reactive ganglioside in the tumor is IV^{3}αNeuAc-Lc$_4$Cer and that DU-PAN-2 has a rather broad specificity.

Key words: DU-PAN-2; Ganglioside; Pancreatic cancer

1. Introduction

DU-PAN-2 monoclonal antibody (mAb) is a prerequisite for the diagnosis of pancreatic cancer in patients with Lewis-negative phenotypes [3–5]. Since the establishment of the mAb in 1982 [6], efforts to determine its epitope have been focused on the glycoprotein in the serum and ascites in pancreatic cancer patients [7,8] or in the cultured cell lines of pancreatic cancer [9]. However, a detailed antigen structure has not, to date, been published. Here we report that gangliosides recognized by DU-PAN-2 in the tumor of a pancreatic cancer patient with Lewis-negative phenotype are IV^{3}αNeuAc-Lc$_4$Cer containing normal and hydroxy fatty acids. In addition, ELISA and TLC-immunostaining with commercially available gangliosides showed that DU-PAN-2 recognizes the IV^{3}αNeuAc-Lc$_4$Cer structure as the most compatible epitope but also shows considerable reactivity with IV^{6}αNeuAc-Lc$_4$Cer and IV^{6}αNeuAc-nLc$_4$Cer.

2. Materials and methods

2.1. Materials

A tumor specimen was obtained during a laparotomy from a 61-year-old female with pancreatic body cancer. Results of laboratory blood examination were: Lewis blood group, Le(a−,b−); CA19-9, 22 U/ml (normal <37 U/ml); DU-PAN-2, 27,000 U/ml (normal <150 U/ml); and sialyl-LeX-i 55 U/ml (normal <38 U/ml). The histopathological diagnosis of the tumor was duct cell carcinoma and moderately differentiated adenocarcinoma. A ganglioside mixture (GM3, GM1 GD1a and GD1b) used as a reference was purchased from Matreya Inc. (PA, USA); chemically synthesized IV^{3}αNeuAc-Lc$_4$Cer, IV^{3}αNeuAc-nLc$_4$Cer, IV^{6}αNeuAc-Lc$_4$Cer and IV^{6}αNeuAc-nLc$_4$Cer were from Wako Pure Chemical Industries (Tokyo); DU-PAN-2 (mouse monoclonal antibody, IgM) was from Kyowa Medix (Tokyo); and biotinylated anti-mouse IgM (μ-chain specific, goat); Vectastain ABC kit and DAB substrate kit for peroxidase were from Vector Laboratories Inc. (Burlingame, CA). Microtiter immunoassay plates (Immuron 1; flat bottom) were purchased from Dynatech Laboratories Inc. (Virginia).

2.2. Extraction and isolation of gangliosides from the tumor

Tumor tissue (18.8 g wet weight) from the patient with pancreatic cancer was homogenized in 60 ml of distilled water with a Warring blender and then extracted with 400 ml of chloroform/methanol (2:1, by vol.), and 200 ml each of chloroform/methanol (1:1, by vol.) and (1:2, by vol.). The combined lipid extract was evaporated to dryness. The lipids were suspended in distilled water, dialyzed against distilled water and dried. Crude lipids were dissolved in chloroform/methanol/water (30:60:8, by vol.) and subjected to DEAE-Toyopearl (25 ml bed volume, acetate form). After neutral lipids had been washed with 300 ml of chloroform/methanol/water (30:60:8, by vol.), the monosialoganglioside fraction was eluted with 150 ml of chloroform/methanol/0.02 M sodium acetate in water (30:60:8, by vol.), the disialoganglioside fraction with 150 ml of chloroform/methanol/0.1 M sodium acetate in water (30:60:8, by vol.), and the polysialoganglioside fraction with chloroform/methanol/1 M sodium acetate in water (30:60:8, by vol.). The three ganglioside fractions were dialyzed against distilled water and lyophilized.

The monosialoganglioside fraction, which contained DU-PAN-2-positive gangliosides, was subjected to alkaline treatment with 0.1 N KOH in methanol for 2 h at 37°C. After neutralization with acetic acid, the fraction was desalted by Sephadex LH20 column chromatography, as described by Nakamura et al. [10]. Then, monosialogangliosides were separated by HPLC (Waters 600E, Millipore Corp., Milford) on a silica gel column (Develosil GPG-010-70H, 4.6 mm i.d. × 250 mm, Nomura Chemical Ltd., Aichi) with programmed gradient elution with isopropanol/*n*-hexane/3 N ammonium hydroxide/water, from 10% of 170:5:15:30 (by vol.) in 165:120:15:0 (by vol.) to 70%, in 15 min [11]. The flow rate was 1 ml per min and oven temperature was kept at 40°C. The eluate was monitored via UV absorption at 210 nm by a UV detector with a flow cell. This HPLC gave 9 fractions. Aliquots of the fractions were monitored by means of TLC and TLC-immunostaining with DU-PAN-2 mAb.

2.3. Permethylation study and fatty acid analysis of isolated gangliosides

The isolated gangliosides were methylated with powdered sodium hydroxide and methyl iodide in dry dimethyl sulfoxide [12,13]. The permethylated gangliosides were purified by Iatrobeads column chromatography [13], then hydrolyzed, reduced and acetylated [14]. The partially methylated alditol acetates were analyzed by GLC on a capillary column of cross-linked 5% phenylmethylsilicone, the temperature being programmed to rise from 170 to 240°C at 2°C/min [15].

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Abbreviations: The nomenclature used for gangliosides is based on the system of Svennerholm [1] and follows recent recommendations [2]. FAB, fast atom bombardment; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody.

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Fatty acid methyl esters recovered with *n*-hexane after methanolysis [16] were analyzed by GLC on a capillary column at 250°C, as previously described [10].

2.4. Mass spectrometry and ^1H NMR spectroscopy of isolated gangliosides

Negative-ion fast atom bombardment (FAB) mass spectrometry of gangliosides was performed with a JEOL JMS-HX110 mass spectrometer equipped with a JMA-DA5000 computer system, with triethanolamine as a matrix. The accelerating voltage was 8.0 kV and primary beam for bombardment was 6.0 kV Xe°.

^1H NMR analysis of the isolated gangliosides was performed in 0.4 ml of dimethyl sulfoxide-d_6 containing 2% D_2O with a JEOL JNM GX-400, 400MHz ^1H NMR spectrometer at 60°C. Tetramethylsilane was used as an internal standard of chemical shifts.

2.5. TLC-immunostaining

The immunostaining was carried out according to the method of Hansson et al. [17] modified to use an avidin–biotin system. The conditions used were DU-PAN-2 mAb (1:100 dilution) and 2 h incubation at room temperature, and biotinylated goat anti-mouse IgM (1:200 dilution) and 1 h incubation at room temperature. Finally bands were detected with Vectastain ABC and diaminobenzidine. Washing after each step was performed with phosphate-buffered saline (PBS).

As an antigen-dilution experiment, the isolated gangliosides and reference gangliosides (IV^{3}αNeuAc-Lc$_4$Cer, IV^{3}αNeuAc-nLc$_4$Cer, IV^{6}αNeuAc-Lc$_4$Cer and IV^{6}αNeuAc-nLc$_4$Cer) from 0.013 to 0.5 μg, were developed on a silica gel plate (HPTLC; Merck) with a solvent system of chloroform/methanol/0.2% aqueous $CaCl_2$ (55:45:8, by vol.). Relative intensities of bands detected on the TLC plates were calculated by densitometric scanning (CS-9000, Shimadzu, Kyoto).

2.6. Enzyme-linked immunosorbent assay (ELISA)

Wells of microtiter plates were each coated with 100 ng of the gangliosides. The wells were incubated with DU-PAN-2 solution (1:12.5 to 1:25600) for 2 h at room temperature, then with biotinylated anti-mouse IgM (1:200 dilution) for 1 h, and then Vectastain ABC solution for 1 h. After each step, the solutions were washed three times with PBS. Peroxidase activity in each well was detected with 50 μl each of 1.27 mM H_2O_2 and 2.5 mM 4-aminoantipyrine in 0.17 M phenol. The absorbance of the developed color was measured at 490 nm after incubation at 37°C for 30 min.

3. Results

3.1. DU-PAN-2 reactive gangliosides of pancreatic cancer

The pancreatic tumor contained GM3 as the major ganglioside and several gangliosides migrating slower than GM3 (Fig. 1A). Of these gangliosides, two bands migrating slower than GM3 and faster than GM1, reacted with DU-PAN-2 mAb (Fig. 1B). The two bands, named gangliosides X and Y, were separated by ion-exchange column chromatography and HPLC. The yields of gangliosides X and Y were approximately 37 μg and 32 μg from 18.8 g wet tissue, respectively. Gangliosides X and Y co-migrated with IV^{3}αNeuAc-Lc$_4$Cer and IV^{3}αNeuAc-nLc$_4$Cer, respectively, but not with IV^{6}αNeuAc-Lc$_4$Cer and IV^{6}αNeuAc-nLc$_4$Cer.

3.2. Structural characterization of DU-PAN-2 reactive gangliosides

In a permethylation study by GLC, both gangliosides were demonstrated to contain: 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylglucitol; 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetylgalactitol; 4,6-di-*O*-methyl-1,3,5-tri-*O*-acetyl-2-deoxy-2-*N*-methylacetamidoglucitol; and 3,6-di-*O*-methyl-1,4,5-tri-*O*-acetyl-2-deoxy-2-*N*-methylacetamidoglucitol in a molar ratio of 1.00:2.13:0.89:0.18 for ganglioside X and 1.00:2.15:0.89:0.05 for ganglioside Y. No other alditol acetates could be observed by GLC. These results indicate that ganglioside X is IV^{3}αNeuAc-Lc$_4$Cer and contains about 18% of ganglioside containing 4-substituted GlcNAc, and ganglioside Y is IV^{3}αNeuAc-Lc$_4$Cer and contains about 5% of ganglioside containing 4-substituted GlcNAc, both of these contaminated gangliosides being possibly IV^{3}αNeuAc-nLc$_4$Cer.

GLC analysis of fatty acids demonstrated that ganglioside X contained C16:0 and C24:1, and ganglioside Y, C16:0 and C24h:1, as the major fatty acids.

As shown in Fig. 2, in the negative-ion FAB mass spectra of gangliosides X and Y, two major pseudo-molecular ions

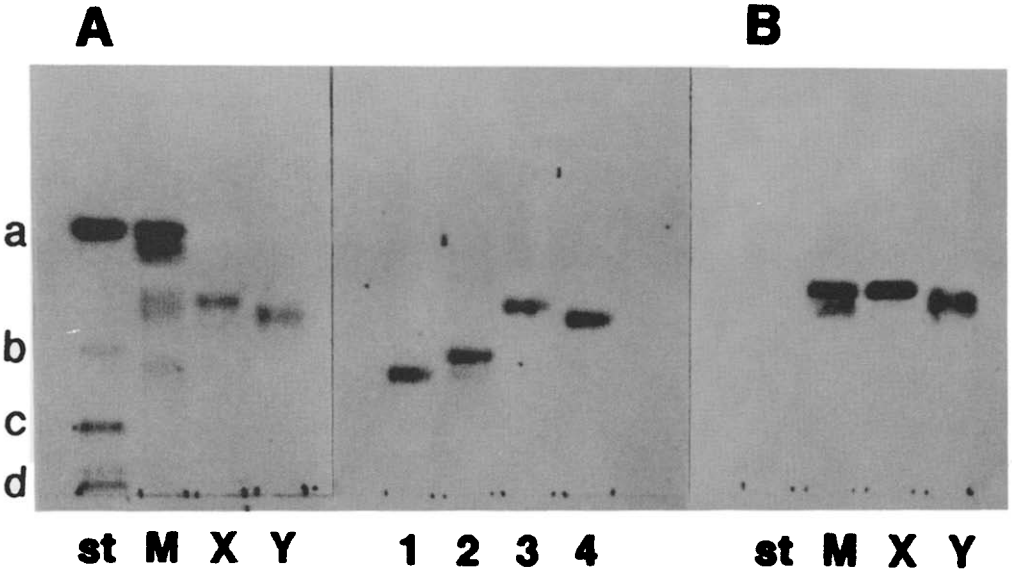

Fig. 1. TLC and TLC-immunostaining of isolated gangliosides from pancreatic cancer. The gangliosides were developed with a solvent system of chloroform/methanol/0.2% aqueous $CaCl_2$ (55:45:8, by vol.). They were detected with resorcinol-HCl reagent (A) and immunostaining with DU-PAN-2 mAb (B). Lanes: st, reference gangliosides, a, GM3; b, GM1; c, GD1a; and d, GD1b; M, monosialogangliosides from pancreatic cancer; X, ganglioside X (0.5 μg); Y, ganglioside Y (0.5 μg); 1, IV^{6}αNeuAc-nLc$_4$Cer; 2, IV^{6}αNeuAc-Lc$_4$Cer; 3, IV^{3}αNeuAc-Lc$_4$Cer; 4, IV^{3}αNeuAc-nLc$_4$Cer.

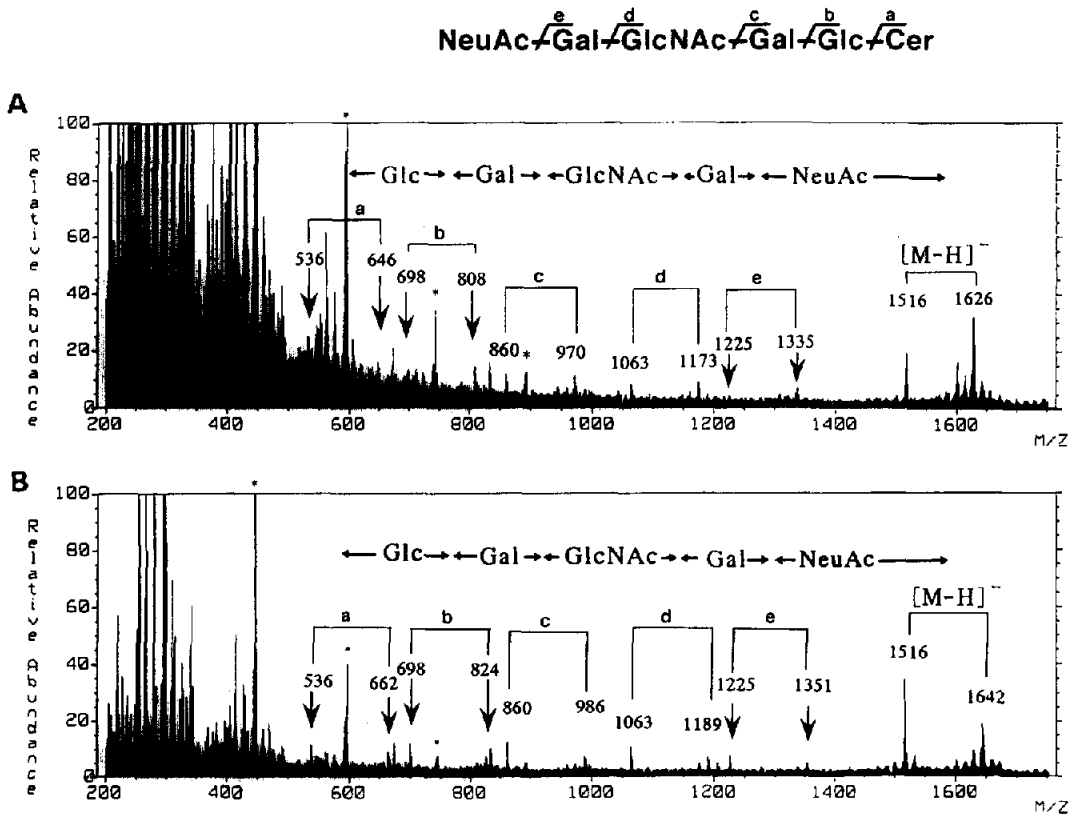

Fig. 2. Negative-ion FAB mass spectra of gangliosides X (A) and Y (B). *, matrix.

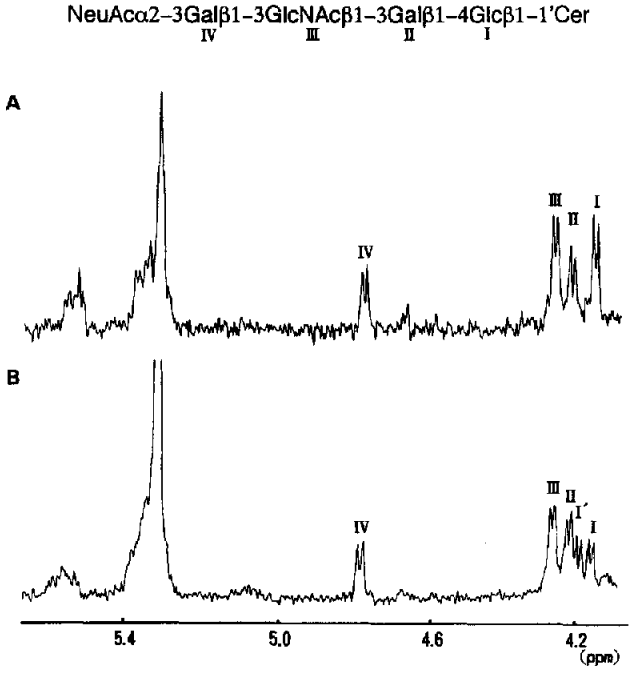

Fig. 3. ^{1}H NMR spectra of gangliosides X (A) and Y (B).

([M-H]$^-$) were detected at *m/z* 1516 and 1626 (Fig. 2A) and 1516 and 1642 (Fig. 2B), which are consistent with the values calculated for the proposed structures with C16:0- and C24:1-C18 sphingenine (Fig. 2A) and C16:0- and C24h:1-C18 sphingenine (Fig. 2B), respectively. In these mass spectra, the ions responsible for the successive elimination of sugar moieties were detected at *m/z* 1225 and 1335 (Fig. 2A) and 1225 and 1351 (Fig. 2B) for ([M-H-NeuAc]$^-$); at *m/z* 1063 and 1173 (Fig. 2A) and 1063 and 1189 (Fig. 2B) for ([M-H-NeuAc-Gal]$^-$); at *m/z* 860 and 970 (Fig. 2A) and 860 and 986 (Fig. 2B) for ([M-H-NeuAc-Gal-GlcNAc]$^-$); at *m/z* 698 and 808 (Fig. 2A) and 698 and 824 (Fig. 2B) for ([M-H-NeuAc-Gal-GlcNAc-Gal]$^-$); and at 536 and 646 (Fig. 2A) and 536 and 662 (Fig. 2B) for ([M-H-NeuAc-Gal-GlcNAc-Gal-Glc]$^-$).

The identification was further supported by the results of ^{1}H NMR spectroscopy, as shown in Fig. 3. In the anomeric proton region of ganglioside X (Fig. 3A), four signals can be seen at 4.18 (J = 8.0 Hz), 4.24 (7.4 Hz), 4.28 (6.7 Hz) and 4.79 (8.0 Hz) ppm, which were identified as anomeric protons of β-glucose (I), β-galactose (II), β-galactose (IV) and β-*N*-acetylglucosamine (III), respectively. In the anomeric proton region of ganglioside Y (Fig. 3B), four of the five anomeric signals which can be seen at the same chemical shift as those of ganglioside X, were identified as β-glucose (I), β-galactose (II), β-galactose (IV) and β-*N*-acetylglucosamine (III) at 4.18 (J = 7.9 Hz), 4.24 (8.0 Hz), 4.28 (6.7 Hz) and 4.79 (8.6 Hz) ppm, respectively. An additional signal observed at 4.21 ppm (8.0 Hz) was identified as β-glucose (I') of the molecule containing α-hydroxy fatty acids (Sako et al. reported that the α-hydroxy group of the fatty

acid moiety affects the chemical shift of the anomeric proton of β-glucose [18,19]).

3.3. Reactivity of gangliosides X and Y, and sialyllacto- and neolacto-tetraosylceramides to DU-PAN-2

On TLC-immunostaining with antigen dilutions, as shown in Fig. 4, ganglioside X and Y and IV^{3}αNeuAc-Lc$_4$Cer exhibit similar binding activity, confirming that the structures of gangliosides X and Y are IV^{3}αNeuAc-Lc$_4$Cer from immunological reactivity. In addition, DU-PAN-2 reacts moderately with IV^{6}αNeuAc-Lc$_4$Cer and IV^{6}αNeuAc-nLc$_4$Cer and significantly less so with IV^{3}αNeuAc-nLc$_4$Cer. In ELISA with antibody dilutions (Fig. 5) gangliosides X and Y exhibit similar binding activities to IV^{3}αNeuAc-Lc$_4$Cer. It was found that IV^{6}αNeuAc-Lc$_4$Cer and IV^{6}αNeuAc-nLc$_4$Cer exhibited moderate reactivity while IV^{3}αNeuAc-nLc$_4$Cer showed little reactivity. On the basis of these results obtained through TLC-immunostaining and ELISA, it is concluded that DU-PAN-2 has a rather broad specificity, reacting with NeuAcα2-6 derivatives as well as IV^{3}αNeuAc-Lc$_4$Cer.

Considering all results, we conclude that the structures of the gangliosides which reacted with DU-PAN-2 in the cancer tissue are IV^{3}αNeuAc-Lc$_4$Cer containing normal and hydroxy fatty acids, and that DU-PAN-2 has a rather broad specificity.

4. Discussion

For one decade, studies into the DU-PAN-2 epitope have been performed using mucin-like molecules from the serum and ascites of patients with pancreatic cancer, or cultured cancer cell lines [8,9,17]. However, the epitope structure has not been elucidated. To date, summarized information is as follows: the epitope (i) is on a mucin-like substance or glycoprotein, (ii) is sensitive to neuramidase and alkaline reduction, (iii) is stable on heating for more than 15 min, (iv) does not react with more than 15 ganglio-series gangliosides, and (v) contains fucose,

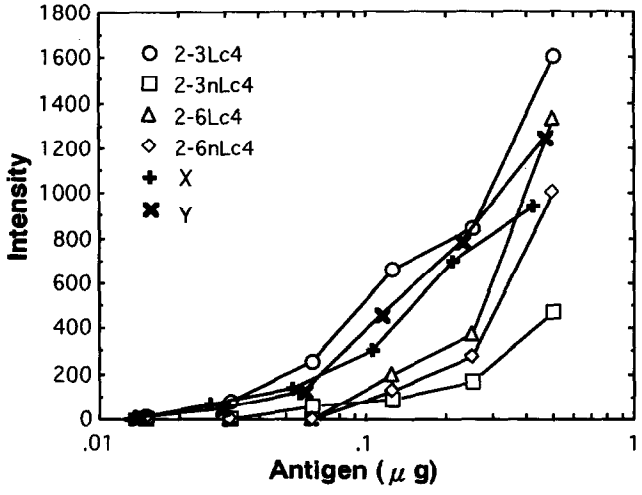

Fig. 4. TLC-immunostaining with antigen dilutions. Various amounts of the gangliosides (2-3Lc$_4$, IV^{3}αNeuAc-Lc$_4$Cer; 2-3nLc$_4$, IV^{3}αNeuAc-nLc$_4$Cer; 2-6Lc$_4$, IV^{6}αNeuAc-Lc$_4$Cer; 2-6nLc$_4$, IV^{6}αNeuAc-nLc$_4$Cer; X, ganglioside X; and Y, ganglioside Y) were applied on a HPTLC plate and developed with a solvent system of chloroform/methanol/0.2% aqueous $CaCl_2$ (55:45:8, by vol.). The bands were detected with DU-PAN-2 mAb (1:200 dilution) and ABC amplification. The intensities of the bands were determined with a densitometer.

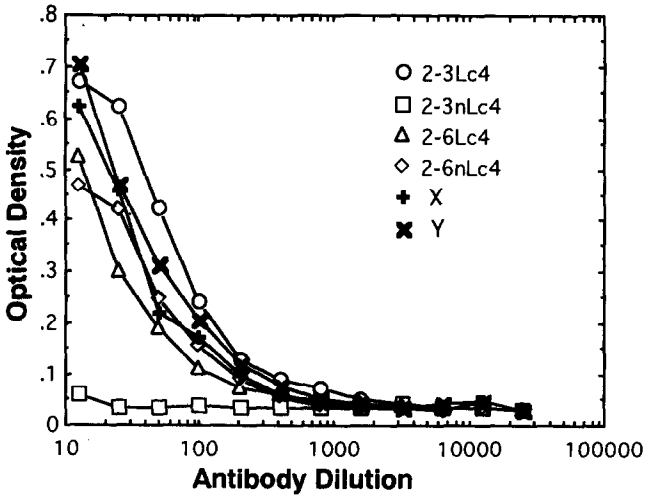

Fig. 5. ELISA with DU-PAN-2 mAb dilution. The gangliosides (2-3Lc$_4$, IV^{3}αNeuAc-Lc$_4$Cer; 2-3nLc$_4$, IV^{3}αNeuAc-nLc$_4$Cer; 2-6Lc$_4$, IV^{6}αNeuAc-Lc$_4$Cer; 2-6nLc$_4$, IV^{6}αNeuAc-nLc$_4$Cer2-6; X, ganglioside X; and Y, ganglioside Y), at 0.1 μg/well, were incubated with a serial dilution of DU-PAN-2 mAb.

galactose, GlcNAc, GalNAc and sialic acid. On the other hand, the fact that the sera of Lewis-negative patients with pancreatic cancer seldom react with CA19-9 mAb but often with DU-PAN-2, allows us to assume that the precursor (defucosyl sialyl Le^a) of sialyl Le^a may be the epitope of DU-PAN-2. In fact, Magnani, in a preliminary report stated that the epitope recognized by DU-PAN-2 antibody was NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc [20].

We obtained pancreatic cancer tissues from a Le(a−,b−) patient exhibiting a high value in the DU-PAN-2 clinical test and were able to confirm that the gangliosides detected with DU-PAN-2 mAb in the cancer are IV^{3}αNeuAc-Lc$_4$Cer containing normal and hydroxy fatty acids. Ganglioside fractions other than the monosialoganglioside fraction did not exhibit any bands detected by DU-PAN-2, confirming the antigen in the cancer tissue to be IV^{3}αNeuAc-Lc$_4$Cer. On TLC immunostaining of ganglioside fractions obtained from the sera of patients exhibiting high values of DU-PAN-2, no bands were detected, indicating that DU-PAN-2 antigen in sera would be carbohydrate chains of glycoproteins but not of gangliosides. Gangliosides contain single carbohydrate chains while glycoproteins have heterogeneous and multiple carbohydrate chains on one molecule, and therefore, in the determination of epitope structure, gangliosides have the advantage.

In our immunological studies, DU-PAN-2 reacted strongly with IV^{3}αNeuAc-Lc$_4$Cer, and moderately with IV^{6}α euAc-Lc$_4$Cer and IV^{6}αNeuAc-nLc$_4$Cer. IV^{3}αNeuAc-nLc$_4$Cer showed little reactivity to the antibody. At present, it is difficult to explain how DU-PAN-2 can react with NeuAcα2-6 derivatives but interesting to note that it does so, especially at high concentrations of antigen. It might be possible that, if we can produce a mAb which reacts with IV^{3}αNeuAc-Lc$_4$Cer in a more specific manner, we can reduce the number of false-positive cases.

Our results for the gangliosides of the pancreatic cancer tissue confirmed that the gangliosides recognized by DU-PAN-2 are IV^{3}αNeuAc-Lc$_4$Cer but not IV^{3}αNeuAc-nLc$_4$Cer, IV^{6}αNeuAc-Lc$_4$Cer or IV^{6}αNeuAc-nLc$_4$Cer.

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