Detection of Patients with Cancer by Monoclonal Antibody Directed to Lactoneotetraosylceramide (Paragloboside)

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ABSTRACT

A hybridoma producing monoclonal antibody (H11) directed to lactoneotetraosylceramide (paragloboside) has been established from spleen cells of a mouse immunized with paragloboside. The monoclonal antibody H11 (immunoglobulin M type) was selected from five clones showing different reactivities with paragloboside. The monoclonal antibody was highly specific to paragloboside and lacked reactivity with other glycolipids including glucosylceramide, lactosylceramide, globotriaosylceramide, globotetraosylceramide, and GaINAcβ1-4[NeuAcα2-3Galβ1-4Glcβ1-1Cer]. However, the monoclonal antibody (H11) was found to bind to lactosamine-containing glycolipids at their terminals, such as i- and l-type glycolipids as well as paragloboside. A two-step sandwich radiomunoassay method for paragloboside antigen in serum was established by using the monoclonal antibody. The mean paragloboside antigen concentration in the sera from 20 normal individuals was 25.3 ng/ml. If the cutoff value was set at 250 ng/ml (Sd = 26.0 + 2 x 27.8 Sd), only 1 of 20 healthy controls had an elevated paragloboside value in the serum, whereas sera from 9 of 12 (75.0%) hepatoma, 4 of 10 (40%) pancreatic cancer, 16 of 40 (40.0%) stomach cancer, and 6 of 10 (60%) lung cancer patients had elevated paragloboside values. Sera from 3 of 8 hepatitis patients and 7 of 10 liver cirrhosis patients were estimated to be positive but sera from 16 patients with benign disease had paragloboside levels lower than the cutoff value. A larger amount of the antigen was found in liver metastases from colorectal carcinoma compared to the normal counterpart. The antigen was also detected in the medium of various human cancer cell lines and meconium. However, the antigen in the sera, medium, meconium, and cancer tissue seemed to be associated with glycoprotein or lipoprotein, because most of the antigen activity was eluted in the void volume fraction on high-performance liquid chromatography with a gel filtration column.

INTRODUCTION

Many monoclonal antibodies prepared by the immunization of mice with human cancer cells have been reported to recognize carbohydrate antigens on tumor cell surfaces (1-9). Some of these monoclonal antibodies are useful for the detection of patients with cancer (1, 5, 9). DelVillano et al. (10) showed that a monoclonal antibody to sialylated Le\textsuperscript{a} (CA 19-9) hapten having type I chain carbohydrate structure reacted to sera of pancreatic cancer patients with high incidence but not to sera from healthy controls. After this report, China et al. (9) demonstrated a different monoclonal antibody directed to sialylated Le\textsuperscript{a} hapten having type II chain structure which was present in pancreatic cancer patients with high incidence but not to sera from healthy controls. In a previous paper (13), we reported the glycolipid composition of ascitic fluids from cancer patients, and paragloboside was found to be a glycolipid which could be detected with high frequency in the fluids from pancreatic cancer and hepatoma. Paragloboside was also demonstrated to be present in various human cancer cell lines in our previous study (14). On the basis of these observations and the fact that paragloboside is a precursor glycolipid of Le\textsuperscript{a} and dimeric Le\textsuperscript{a} hapten, we prepared monoclonal antibody directed to paragloboside in order to detect the antigen in sera from patients with cancer.

In the present paper we describe the preparation of monoclonal antibody against paragloboside, the establishment of an assay method for paragloboside by two-step sandwich RIA,\textsuperscript{2} and the detection of the antigen in sera from cancer patients with high incidence.

MATERIALS AND METHODS

Monoclonal Antibody H11 Directed to Paragloboside. Paragloboside was prepared from sialosylparagloboside of bovine erythrocyte (12) by mild acid hydrolysis (15). The hydrolysate was dialyzed against distilled water and lyophilized. Paragloboside thus obtained was purified by Iatrobeads column chromatography as described previously (14). Preparation of monoclonal antibody directed to paragloboside was performed according to the method described by Young et al. (16). The mixture of paragloboside and acid-treated Salmonella minnesota was injected i.p. into BALB/c mice six times at 2-week intervals. Ten days after the sixth injection, 50 µg of paragloboside were injected i.p. and on the third day after the last injection, spleen cells were harvested and fused with P3U1 mouse myeloma cells. The hybridomas secreting antibody against paragloboside were selected by enzyme immunoassay using paragloboside-coated microtiter plates. By this procedure, five hybridomas secreting antibody directed to paragloboside were obtained. Based on the reactivity with paragloboside and the specificity, monoclonal antibody H11 (IgM type) was used in the present study.

Detection of Antibody against Paragloboside by Enzyme Immunoassay. Indirect solid phase enzyme immunoassay was used to detect antibody against paragloboside. Paragloboside (0.1 µg/ml) in ethanol solution was delivered into each well of a flat-bottomed 96-well polystyrene plate (Tittertek; Flow Laboratories, Inc.) and evaporated under reduced pressure. Then 100 µl of 0.3 M sucrose solution were added to each well and left for 10 min at room temperature. After removal of the sucrose solution by suction, 100 µl/well of hybridoma culture medium were added and the plate was incubated for 60 min at room temperature. After the reaction, the mixture was removed by suction and the wells were washed with 100 µl/well of PBS (pH 7.3). Horseradish peroxidase-conjugated anti-mouse immunoglobulin antibody (purchased from Cappel Laboratories) (50 µl) was added to each well and the plate was incubated at room temperature for 60 min with shaking and then washed with 0.05% Tween 20. Next, 100 µl per well of o-phenylenediamine solution were added and the plate was incubated at room temperature for 10 min. Finally, the mixture was removed by suction and the wells were washed with 0.05% Tween 20, 1512

\textsuperscript{2} The abbreviations used are: RIA, radioimmunoassay; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; GlcCer, Glcβ1-1Cer; LacCer, Galβ1-4Glcβ1-Cer; GbCer, Gal-1-Galβ1-4Glcβ1-Cer; GbCer, Gal-NAcβ1-3Galβ1-4Glcβ1-Cer; GalCer, GalNAcβ1-4Galβ1-4Glcβ1-Cer; Gb3Cer, GalNAcβ1-3Galβ1-4Glcβ1-Cer; Galβ1-4Glcβ1-Cer; Gb4Cer, GalNAcβ1-4Galβ1-4Glcβ1-Cer; Gb5Cer, GalNAcβ1-3Galβ1-4Glcβ1-Cer; I-glycolipid, Galβ1-4Glcβ1-Cer; I-glycolipid, Galβ1-4Glcβ1-Cer; I-glycolipid, Galβ1-4Glcβ1-Cer; I-glycolipid, Galβ1-4Glcβ1-Cer; I-glycolipid, Galβ1-4Glcβ1-Cer; I-glycolipid, Galβ1-4Glcβ1-Cer; I-glycolipid, Galβ1-4Glcβ1-Cer; I-glycolipid, Galβ1-4Glcβ1-Cer;

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temperature for 15 min. The reaction was stopped by the addition of 100 μl of 1 N H₂SO₄ per well. The color development was read at 490 nm.

Two-Step Sandwich RIA. Immunoglobulin M (H11) obtained from BALB/c mice was subjected to HPLC on a Superose 6 column (1.2 x 30 cm, purchased from Pharmacia) and eluted with phosphate-buffered saline (pH 7.2) at a flow rate of 0.3 ml/min. The purified IgM was radiiodinated with chloramine-T reagent (17). The specific activity of the ¹²⁵I-labeled H11 antibody was adjusted to 5 g iodine/g immunoglobulin. Polystyrene beads (0.25 inch in diameter; Precision Plastic Ball Co., Chicago, IL) were coated with the purified antibody and dried (10).

Two-step sandwich RIA was performed as follows. An anti-paragloboside monoclonal antibody-coated bead was incubated with 100 μl of sample serum (20-fold diluted with PBS before use) or paragloboside standard solution (buffer A, PBS containing 5% horse serum and 0.1% NaN₃) and 100 μl of buffer A at room temperature for 20 h in a 60-well plastic plate with shaking. The bead was washed three times with PBS; then 200 μl of ¹²⁵I-anti-paragloboside monoclonal antibody (213,472 cpm) were added and the mixture was incubated at room temperature for 20 h in the plate with shaking. The bead was washed with PBS and the radioactivity bound on the bead was counted with a gamma counter.

Immuno-staining of Paragloboside on Thin Layer Plate. The specificity of the monoclonal antibody (H11) was determined by immunostaining on a TLC plate by the method described previously (14). The developed color was determined by chromatocanmer (Shimadzu CS 910).

High Performance Liquid Chromatography. Isolation of paragloboside antigen from serum, meconium, culture medium of cancer cells, and cancer tissue was performed by HPLC on a TSK G 400 SW column (7.5 mm inside diameter x 60 cm; Toyo Soda Co., Ltd.). The excluded molecular weight range of the column is 7 x 10⁴ (maximum) to 1 x 10⁶ (minimum). Preparation of samples applied to HPLC was as follows. Each serum from healthy controls or patients with gastric cancer, liver cirrhosis, and hepatoma was centrifuged at 15,000 x g for 10 min. The supernatant was filtered with membrane filter (0.45 pm pore size). A 100-μl sample of the filtrate was applied to HPLC. In the case of paragloboside-added serum, 100 μg of paragloboside were mixed with 1 ml of serum from healthy controls and then centrifuged at 15,000 x g for 10 min. The other method was the same as mentioned above. Tissue of liver metastases from colon cancer was extracted with 3 volumes of PBS, treated at 80°C for 10 min, and centrifuged at 15,000 x g for 10 min. The supernatant was filtered with the membrane filter and 100 μl of the filtrate was applied to the HPLC. Meconium was extracted with 3 volumes of PBS and the extract was centrifuged at 15,000 x g for 10 min. The supernatant was filtered with the membrane filter and 100 μl of the filtrate were applied to the HPLC. Culture supernatant of AZ 521 cells was filtered with the membrane filter. A 100-μl sample of the filtrate was applied to the HPLC. The column was eluted with PBS containing 0.1% NaN₃ at a flow rate of 0.7 ml/min using a Waters M 600 system. Each fraction was monitored by two-step sandwich RIA to detect paragloboside antigen.

RESULTS

Specificity of H11 Monoclonal Antibody. The specificity of H11 monoclonal antibody directed to paragloboside was tested by using two-step sandwich RIA. As antigen glycolipids, concentrations of 10, 1, and 0.1 μg/ml of GlcCer, LacCer, GbCer, GbCer, GgCer, GgCer, GM₁, and ceramide were used. The antibody failed to react with all the glycolipids used except for paragloboside (Table 1). In addition to these various glycolipids, binding activity of H11 to lactosamine-containing glycolipids was studied by immunostaining technique on a TLC plate. For this study, i- and I-type glycolipids were tested as well as LacCer (as negative control) and paragloboside (as positive control). GM₁, sialylparagloboside, and i- and I-type gangliosides, which had been prepared from human placenta, were treated with Cl. perfringens neuraminidase and separated by TLC. Various amounts of these asialoligandosides, LacCer, paragloboside, and i- and I-type glycolipids were subjected to immunostaining analysis and the developed color was determined by chromatocanmer (Fig. 1a). The result was summarized in Fig. 1b. LacCer showed no binding activity. Three lactosamine-containing glycolipids, paragloboside, and i- and I-type glycolipids showed almost same binding activity to the H11 monoclonal antibody. Le⁴ glycolipid did not show the binding activity to H11 (data not shown). From these results, H11 was found to recognize the terminal lactosamine structure of glycolipids.

Two-Step Sandwich RIA. A typical standard curve of paragloboside obtained by two-step sandwich RIA is shown in Fig. 2a. The curve was essentially linear from 0.5 to 117 ng/ml. The minimum paragloboside concentration detected by the RIA was 100 pg/ml. In order to determine the stability of antibody-coated beads, we tested beads prepared freshly, or 1 month after being stored in the refrigerator.
PARAGLOBOSIDE, A TUMOR-ASSOCIATED GLYCOLIPID

Fig. 2. a, dose-response curve for paragloboside antigen obtained by the two-step sandwich RIA. O — O, monoclonal antibody-coated beads freshly prepared for use, • — •, monoclonal antibody-coated beads prepared 1 month before use. O — O, monoclonal antibody-coated beads prepared 2 months before use.

Analytical recovery of paragloboside was determined by diluting samples with pooled human serum and also serum containing a very low concentration of paragloboside and then assaying them by the RIA. The recovery of paragloboside was 75.5 ± 9.0% (SD) of the expected value, indicating relatively good assay recovery of the antigen after dilution with normal human serum. Serial dilutions of each serum were examined using the RIA. When the sera were diluted to more than 20-fold, the plots became approximately linear (Fig. 3).

Paragloboside in Serum of Patients with Cancer or Benign Diseases. The distribution of paragloboside values in sera from patients with cancer or benign disease is shown in Fig. 4. Paragloboside was detectable in 75% of sera from normal healthy controls but 25% were negative. Based on the concentration of paragloboside in control sera, the cutoff value was determined to be 80.9 ng/ml (mean value ± 2 SD; n = 20). Among the cancer patients, paragloboside concentrations higher than the cutoff value were observed in 75.0% of those with hepatoma and 40.0% of patients with pancreatic cancer. Further, 60.0% of patients with lung cancer and 40.0% of patients with gastric cancer had increased paragloboside values. Among liver disease patients, 37.5% of hepatitis patients and 70% of liver cirrhosis patients gave higher amounts of paragloboside than the cutoff value, but all sera from other benign diseases showed values less than 80.9 ng/ml.

Detection of Paragloboside Antigen in Cancer Tissue, Meconium, and Culture Medium of Cancer Cells. Based on the detection of paragloboside antigen in sera from cancer patients with high incidence, we tried to detect paragloboside in tumor tissue, in meconium, and also in culture medium of cancer cells. Table 2 shows the paragloboside concentrations found in these samples. Very high concentrations of paragloboside antigen were detected in liver metastases from colorectal carcinoma compared to the normal counterpart except for one case. The antigen concentration found in the cancer tissue was 7 to 73 times higher than that of normal tissues.

We detected large amounts of paragloboside antigen in human meconium, as shown in Table 2. Further, we tested eight culture media derived from various cell lines. Paragloboside...
was detected in all culture media at various concentrations. In the medium of AZ 521, a gastric adenocarcinoma cell line, more than 333 ng/ml of paragloboside antigen were detected.

Paragloboside Antigen in Sera, Cancer Tissue, and Meconium. Glycolipid antigens defined by monoclonal antibodies are sometimes expressed as glycoprotein-associated forms rather than as the glycolipid form in serum or on cell surfaces (3, 18, 19). Therefore, we tried to isolate the paragloboside antigen from cancer tissue, meconium, cell culture medium, and serum by HPLC using a gel filtration column. As shown in Fig. 5a, no paragloboside antigen was detected in the eluate from serum of a normal healthy control. When paragloboside was added to the serum, paragloboside was detected after 30 min, and a small peak was also found just before 20 min (Fig. 5b). In all other cases, most of the paragloboside antigen was detected in the fraction just before 20 min but not in the fraction eluted at 30 min (Fig. 5, c-g). The former fraction corresponds to the void volume of the column used. From these results, paragloboside antigen seemed to be present as glycoprotein or lipoprotein form in cancer tissue and body fluids.

**DISCUSSION**

In the previous papers, we proposed that paragloboside was a possible cancer-associated glycolipid because it was found in ascitic fluids from patients with hepatoma and pancreatic cancer and was shown to be expressed on tumor cell surfaces (13, 14). These results suggested that paragloboside might be found in sera of cancer patients with high incidence. This particular glycolipid is a very minor component in normal tissues, although it is found in human erythrocytes and neutrophils (20, 21). Even in erythrocytes and neutrophils, however, the amount of paragloboside is very low. Thus, we tried to assay paragloboside with high specificity and high sensitivity in order to examine its availability as a marker for the detection of cancer-bearing patients. Monoclonal antibody directed to paragloboside prepared for this purpose was of IgM type and was specific to paragloboside. This antibody was used to develop a two-step sandwich RIA which was able to measure 1–300 ng/ml paragloboside; this sensitivity is sufficient to measure paragloboside in human serum (the concentration of the antigen in normal serum is less than 80 ng/ml). By using this RIA method, paragloboside antigen was assayed in sera from patients with cancer and benign diseases. The antigen was detected in the sera from hepatoma and from pancreatic cancer patients with high frequency. The positive incidence of this antigen suggested that this assay is applicable to the detection of patients with cancer; however, the antigen was also recognized in the sera from patients with liver diseases. Paragloboside is a carbohydrate antigen which has galactose as the terminal sugar component.

Asialoglycoproteins are well known to be adsorbed at liver cells, which have receptor sites for the terminal galactose moiety of glycoconjugates (22–24). High concentrations of paragloboside antigen found in sera of patients with hepatoma and liver diseases may indicate that receptors for naked galactose of glycoconjugates are destroyed by these liver diseases, so that the paragloboside antigens circulate without adsorption. This may be why we could detect paragloboside antigen with high frequency in the sera from patients with liver diseases.

In the HPLC experiment, most of the paragloboside antigen was found in the void volume on the gel filtration column. This result suggests either that paragloboside is present as lipoprotein or that the oligosaccharide moiety of paragloboside is associated with glycoprotein. The second possibility seems likely because many carbohydrate antigens defined by monoclonal antibodies were reported to be present as glycoprotein-associated forms (18, 19). However, the first possibility cannot

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**Fig. 5.** Gel chromatography profiles of paragloboside antigen. High-performance liquid chromatography on a TSK G 400 SW column was performed as described in “Materials and Methods.” Fractions were monitored for antigen by the two-step sandwich RIA using monoclonal antibody to paragloboside. a, serum from normal healthy control; b, normal serum with added paragloboside antigen; c, serum from a patient with stomach cancer; d, extract of liver metastases from colorectal carcinoma; e, extract of meconium; f, culture medium of AZ 521 (stomach cancer); g, serum from a patient with liver cirrhosis. Arrows, elution position of marker proteins. 0, void volume; 1, glutamate dehydrogenase (M, 290,000); 2, lactate dehydrogenase (M, 142,000); 3, enolase (M, 67,000); 4, adenylate kinase (M, 32,000); 5, cytochrome c (M, 12,400).
be ruled out because we have isolated paragloboside from ascitic fluid from cancer patients (13) and the majority of glycolipids in serum exist in association with circulating lipoproteins (25-28). When paragloboside was added to the serum from a healthy control, two peaks were detected by RIA. One was in the fraction eluted at 30 min and the other was in the void volume, in which most of the antigen from cancer tissue, meconium, and culture media of cancer cells was detected. This result suggests that a part of paragloboside added to the serum could be adsorbed lipoproteins.

The present data indicate that the two-step sandwich RIA for paragloboside may be useful for detection of patients with hepatoma and pancreatic cancer. However, this method cannot distinguish between cancer and liver diseases. In order to discriminate these diseases, combination with other diagnostic assays should be introduced. Such combination systems are being examined in our laboratory now.

REFERENCES

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