Recent Studies of Glycolipid and Glycoprotein Profiles and Characterization of the Major Glycolipid Antigen in Gastric Cancer of a Patient of Blood Group Genotype pp (Tj"⁻") First Studied in 1951¹

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ABSTRACT

The lyophilized gastric tumor tissue (20 mg) of the patient (D. J.) studied serologically in 1951 absorbed 16 to 32 agglutinating units of her own stimulated antibody (1:512) induced by a test injection of 25 ml of incompatible blood from an initial 1:4 to 1:8 titer. When in 1975 the lyophilized tissue (about 3.5 g) was located, it was mailed to Seattle for biochemical analysis of the antigens involved, and these investigations were not completed until 1982. This paper describes the glycolipids and glycoproteins in the gastric cancer tissue of a patient whose erythrocytes contained the first example of a very rare variant of the P system, identified as genotype pp (initially referred to as Tj"⁻") and later shown to contain anti-P,PPk (anti-Tj") in her serum. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the immunoprecipitates with anti-globoside, anti-P, and anti-P,PPk of [³H]galactose-labeled tissue extract demonstrated the major component in glycolipids and a few minor components in glycoproteins. The purified glycolipid fraction (upper neutral glycolipid) showed complement fixation with anti-P,PPk; nevertheless, the glycolipid fraction contained neither globoside nor ceramide trihexoside. The major glycolipid had the same thin-layer chromatography mobility and antigenic reactivity as a new "X千古 glycolipid" of human erythrocytes which cross-reacts with anti-globoside, anti-P, and anti-P,PPk of [³H]galactose-labeled tissue extract. The latter is a glycolipid with the same terminal structure as globoside and with an internal structure identical to paragloboside. Although the purified glycolipid fractions display a clear inhibition of anti-P, agglutinins, only a minor component had the same thin-layer chromatography mobility as P, which is a ceramide pentasaccharide susceptible to hydrolysis with fig α-galactosidase. It is possible that some other glycolipid having a more complex structure may also have P, activity. The results indicate that the tumor activated the synthesis of P, antigen, but not the synthesis of the globo series glycolipid; it also showed an enhanced synthesis in the lacto series glycolipid with the same terminal structure as globoside. There are indications that this patient survived for 22 years, until 1973, when she died at age 63.

INTRODUCTION

In 1951, the unusual case of the 66-year-old patient (D. J.) with gastric carcinoma was reported, the details of which are summarized in Table 1. Her erythrocytes were shown to be the first example of a blood group antigen in a tumor which is foreign to the host. When Hakomori et al. (7) reported on the globoside-Fs (Forssman) change from normal mucosa to the additional sugar determinant in the mucosa of the adjacent malignant tissue, this led Levine (12) to the self-nonself concept in the cancer. Patient D. J. was in Group O, and her serum contained antibodies which agglutinated all random erythrocytes except her own. Several years later, her erythrocytes were found to be the first variant of the P system, i.e., of genotype pp, which has an incidence of 1:150,000; in her family, however, the incidence was 1:4 because her parents were double first cousins and her younger sister was found also to be of genotype pp and compatible with her sister's antibodies (11, 13). The antibodies in the sera of both the patient and her sister were identified as anti-P,PPk, initially called anti-Tj". A biological test in the form of a minitransfusion of 25 ml of incompatible blood prior to the surgical procedure caused a severe hemolytic reaction, fever, and an increase of her initial 1:4 to 1:8 titer to 1:512. Shortly after her mild transfusion reaction, D. J. submitted to subtotal gastrectomy, from which she recovered completely. At no time during her survival for 22 years until her death from old age in 1973 in her 88th year was there any evidence of metastasis. This is in striking contrast to the outcome in her compatible pp younger sibling who developed cancer of the uterus and died in 1964 with widespread metastasis at the age of 63. The significance of these striking events on this rare family with a built-in control will be discussed more fully by one of us (Philip Levine).⁴

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glycolipids and glycoproteins, which are now reported in this paper.

MATERIALS AND METHODS

Of the total amount of 3.5 g, 500 mg was used for glycoprotein examination as described below, and the remaining material was used for immunological and biochemical characterization of its glycolipids.

Preparation and Analysis of Glycolipids. Tumor powder (1.5 g) was homogenized with 100 ml of chloroform:methanol (2:1) and filtered. The residue was reextracted with 100 ml of chloroform: methanol:water (5:5:1) and refiltered. The combined extracts were evaporated to dryness, dissolved in chloroform:methanol (2:1), and filtered through Celite layers, and the filtrate was evaporated and redissolved in 24 ml of chloroform:methanol (2:1). To this, 4 ml of water were added to perform the partition of Foch et al. (2). The lower-layer fraction was washed twice with chloroform:methanol:0.1% sodium chloride (1:10:10, v/v), and the combined upper layer was dialyzed against water, lyophilized, and separated into gangliosides and neutral glycolipids by DEAE-Sephadex chromatography according to the method of Yu and Ledeen (23). Implications for immunotherapy by monoclonal antibodies specific for ABO-P antigen or their similar (cross-reacting) antigen which is foreign to the host. Tumor is rejected by long-lived, high-titered IgG antibodies specific for the illegitimate glycolipid P, antigen in the cancer.

The abbreviations used in this study: TLC, thin-layer chromatography; ceramide trihexoside, globotriaosylceramide, Galα1–3Galβ1–4Glcβ1–1Cer; globoside, globotetraosylceramide, GalNAcβ1–3Galβ1–4Glcβ1–1Cer; paragloboside, lacto-N-tetraosylceramide, Galβ1–4Glcβ1–1Cer; H, glycolipid, Fuca1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1Cer; Ht, glycolipid, Fuca1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1Cer; H2, glycolipid, Fuca1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1Cer; H3, glycolipid, Fuca1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1Cer; CDH, glycolipid, Fuca1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1Cer; nortiexaosylceramide, Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1Cer; CD, glycolipid, Fuca1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1Cer; DS, ceramide dihexoside, Galβ1–4Glcβ1–1Cer; CD, glycolipid, Fuca1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1Cer; nortiexaosylceramide, lacto-N-tetraosylceramide, Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1Cer.

RESULTS

SDS-Polyacrylamide Gel Electrophoresis Pattern of the Total Immune Precipitate of the Detergent Extract of 3H-Labeled Tissue. In order to obtain information on the distribution of antigens in the glycolipid and glycoprotein fractions, the total immune precipitates of the detergent extract of the 3H-labeled tissue were analyzed in SDS:gel electrophoresis in which the glycolipid-associated activity is detected in the gel front and the glycoprotein-associated activity is found within the gel. As shown in Chart 1, the major activity precipitated with anti-globoside, anti-Pi, and anti-P,PPk was found to be associated with the glycolipid. A clear peak with a high-molecular-weight range and a minor peak with a lower-molecular-weight range was associated with anti-Pi antibodies. Two glycoprotein peaks were associated with the immune precipitate by anti-globoside.

Antigenic Activity of Glycolipid Fractions. To further confirm the activities associated with glycolipids, purified glycolipid fractions were analyzed with anti-P, and anti-P,PPk antibodies. Clear activities were demonstrated in the upper immunological activities were tested by inhibition of agglutination caused by anti-P, and anti-P,PPk. In addition, the major glycolipid present in the upper neutral fraction was further analyzed by the reactivity of the glycolipid on TLC with a few monoclonal antibodies, the specificity of which is defined under "Results."

Thus, with this built-in control, the difference in outcome depended upon the genetic mutation resulted in synthesis of Pi antigen or their similar (cross-reacting) antigen which is foreign to the host. Tumor is rejected by long-lived, high-titered IgG antibodies specific for the illegitimate glycolipid P, antigen in the cancer.

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neutral glycolipid fraction with antibodies to P1 and anti-P1P1P1.
The upper neutral glycolipid (12.5 µg/well) and total gangliosides (12.5 µg/well) completely inhibited agglutination of P1 erythrocytes caused by 3 agglutinating doses of anti-P1. The lower neutral glycolipid (50 µg/well) did not inhibit P1 under the same condition. The upper neutral glycolipid (6.2 µg/well) partially inhibited agglutination of P1 erythrocytes with anti-P1P1P1 (5–1450). Ganglioside fraction had anti-complement activity, and therefore no satisfactory data could be reported.

**Glycolipid Profile in TLC Analysis.** A puzzling finding was that no trace amount of globoside (P antigen) nor ceramide trihexoside (P1 antigen) was found in the glycolipid fraction of the tumor on TLC analysis (Fig. 1) despite the fact that the glycolipid fraction showed a clear activity with anti-globoside. A small quantity of a glycolipid (Band 2) with the same TLC migration as P1 glycolipid was detected (indicated by arrow in Fig. 1, Lanes 2 and 3), but the quantity was insufficient for further characterization. The Band 2 glycolipid having the same mobility as P1 glycolipid and the Band 5 glycolipid were degraded by incubation with a-galactosidase in the presence of sodium deoxycholesterol under the condition described (6), whereby free galactose was liberated.

A large quantity of lactosylceramide (Fig. 1, Double Band 1) and absence of ceramide trihexoside and globoside are a characteristic pattern which is similar to the glycolipid pattern of pp erythrocytes as reported by Marcus et al. (16). The glycolipid (Band 3) was found as the major component (about 250 µg/g dry tissue), which was present mainly in the upper phase (Fig. 1, Lane 2). Smaller quantities were also found in the lower phase if Folch's partition is not exhaustive (Fig. 3, Lanes 5 and 6). Other minor components (Bands 4 and 5) and a relatively major component (Band 6) were separated.

Gangliosides of the tumor also showed a striking difference from that of pp erythrocytes. Sialosylparagloboside in the tumor was only a minor component, while Gd3 was the major component. As was well documented (15, 16), sialosylparagloboside was the major component of pp erythrocytes. Also, there were a few unidentified, slow-migrating gangliosides in the tumor.

**Characterization of a New Glycolipid (x2) of Human Erythrocytes Cross-Reacting with Anti-Globoside and the Identity of the Major No. 3 Band Glycolipid (Fig. 1) of the Tumor**

**Tissue with the x2-Glycolipid.** A very minor glycolipid (x2 component) of human erythrocytes cross-reacting with anti-globoside was isolated and characterized by the procedures as described in "Materials and Methods." The structure was identified as seen below in comparison with paragloboside and globoside.

Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 1Cer = paragloboside

GalNacβ1 → 3Galα1 → 4Galβ1 → 4Glcβ1 → 1Cer = globoside

GalNacβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 1Cer = x2 component (9)

The x2 component has the same terminal structure as globoside and the same internal structure as paragloboside. The major glycolipid of the tumor tissue Band 3 glycolipid (Fig. 1) was found to be identical to x2 glycolipid of erythrocytes based on the following findings: (a) both Band 3 glycolipid and x2 had the same mobility on TLC; (b) acetylated derivatives of both Band 3 and x2 had equally low mobility on TLC, comparable to the acetate of a ceramide hexa- or heptasaccharide (Fig. 2); (c) both x2 and Band 3 glycolipid were degraded by endo-β-galactosidase to give the same pattern of oligosaccharide and glycolipid; (d) both x2 and Band 3 glycolipid were degraded by β-N-acetylhexasaminidase; x2 was converted to paragloboside and Band 3 glycolipid was converted to a glycolipid with the same TLC mobility as paragloboside; (e) both x2 and Band 3 glycolipid were stained on TLC with an IgM monoclonal antibody directed to asialo Gd2 (GalNacβ1 → 4Galβ1 → 4Glcβ1 → 1Cer)7 (Fig. 3).

**DISCUSSION**

The findings in the gastric cancer patient described by Levine et al. (13) suggested a possible appearance of "illegitimate" P1, P, P1 antigen in gastric cancer of a patient with blood group p and or genotype pp. Since these antigens have been established by Marcus et al. (15, 16) as glycolipids with a defined sugar sequence and linkages, i.e., P for Galα1 → 4Galβ1 → 4GlcNAc, P for GalNacβ1 → 3Galα1 → 4Gal, and P for Galα1 → 4Galβ1 → 4Glc, we have examined whether any structure in glycolipids and glycoproteins responsible for these specificities are present in that tumor tissue. Unfortunately the amount of lyophilized tumor tissue available was highly limited, and the glycolipid content of the lyophilized tissue was found to be very low as compared to erythrocytes and fibroblasts, perhaps due to a larger proportion of connective tissue in the tumor. Therefore, a characterization of antigens is necessarily far from complete. Two approaches have been applied. (a) Lyophilized tissue was labeled with galactose oxidase and NaB3H4 (4), through which all terminal galactose and GalNAc residues were labeled, and tissue was extracted.

7 Radioluminunostaining of glycolipid on TLC plate originally described by Magnani et al. (14) requires a very high antibody titer. If the staining is performed with low-titer antibodies, the background staining is too high to reveal any glycolipid "spot." The staining of glycolipid spot on TLC also depends on the affinity of antibodies applied. Antigloboside antibodies failed to stain globoside and X2 glycolipid on TLC plate; on the other hand, IgM monoclonal antibody to asialo Gd2 (22) reacted not only to the original antigen (asialo Gd2) but also to x2 glycolipid, although it did not react to globoside on TLC plate and in precipitation. Therefore, this IgM antibody appears to recognize the structure GalNacβ1 → 3Galβ1 → 4GlcNAc as well as GalNacβ1 → 4Galβ1 → 4Glc but does not recognize the structure GalNacβ1 → 3Galα1 → R. The terminal GalNAc linkage to the penultimate sugar residue could be either β1 → 3 or β1 → 4, but the penultimate sugar linkage must be β1 → 4 (manuscript in preparation).
immunoprecipitated, and analyzed on SDS:gel electrophoresis. (b) Three glycolipid fractions, the upper and the lower neutral glycolipid from the partition of Folch’s et al. (2) and ganglioside, were prepared, and TLC and antigenicity of each glycolipid fraction were compared with that of P, P, and P antigen and that antigen cross-reacting with anti-globoside (x2 glycolipid). The major findings are summarized as follows. (a) The major labeled antigens in the glycolipid fraction were precipitated with 3 antibodies, anti-globoside, anti-P, and anti-P,PPk. (b) The upper neutral glycolipid fraction but not the lower glycolipid fraction inhibited anti-P, agglutination caused by anti-P, and anti-P,PPk, and the upper neutral glycolipid fraction fixed complement with anti-P,PPk. (c) Three glycolipids were present in the upper neutral fraction, and these are corresponding, respectively, to lactosylceramide (the major), P, (the minor), and an unknown major band (Band 3). The major Band 3 glycolipid was indistinguishable from a new erythrocyte glycolipid (x2) having the sugar structure GalNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc → Ceramide that cross-reacts with anti-globoside and anti-asialo GM1 IgM monoclonal antibodies. (d) These glycolipid fractions contained neither globoside (P) nor ceramide trihexoside (P3). The results indicate the following possibilities. (a) Synthesis of P, determinant was induced in the tumor, as was evidenced by the presence of a clear P, reactivity in glycolipid as well as in glycoprotein. P, Active glycolipid may not only be a ceramide pentasaccharide, as identified previously (15,16), but also some glycolipid with longer carbohydrate chain and with sialic acid residue (ganglioside). (b) Synthesis of a glycolipid cross-reacting with anti-globoside antibodies and having a structure GalNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 1Cer. This glycolipid is present in very small quantity in normal erythrocytes (9). The presence of this glycolipid in high concentration in this tumor provides a cross-reactivity with anti-globoside antibodies and possibly with anti-P antibodies. (c) A low level of paragloboside and sialosylparagloboside in the tumor as compared to p-erythrocytes may reflect a high rate in conversion of paragloboside to P-like antigen (β-GalNAc-paragloboside). Although we had no opportunity for analyzing normal gastric mucosa of pp individuals, the glycolipid composition of stomach tissue of an individual with pp phenotype was recently reported (1). They observed that globoside and ceramide trihexoside were also absent in stomach tissue as in erythrocytes. A slowly migrating glycolipid spot was seen in their published TLC picture but was not identified. It is possible that P-like antigen may also be found in normal tissue of pp individuals, but the quantity in the tumor of this case must be much higher. The presence of P, antigen in this tumor must be quite unique. P-like antigen may also be found in normal tissue of pp individuals as well, but the quantity in the tumor must be much higher. A possible mechanism for the expression of these antigens in the tumor is proposed in Chart 2.

REFERENCES


Fig. 1. Thin-layer chromatography of the glycolipid fraction isolated from the tumor of a patient with blood group pp genotype (D. J.). Solvent system used was chloroform:methanol:water (60:35:8, v/v). Visualized with orcinol reagent. Lane 1, reference glycolipids: CMH, CDH, CTH, Glob, and RP indicate, respectively, ceramide monohexoside, ceramide dihexoside, ceramide trihexoside, globoside, and ceramide pentasaccharide with the same sugar sequence as P, antigen purified from rabbit erythrocytes. Lane 2, the neutral glycolipids of the tumor in aqueous layer of Folch's partition; Lane 3, the glycolipids of the tumor in organic layer of Folch's partition. Double spots indicated as No. 1 are the major glycolipids present in organic layer, which are both ceramide dihexose. Band 2, P, glycolipid. Band 3, major glycolipid cross-reacting with anti-globoside antibodies (x2 glycolipid). Bands 4, 5, and 6 are unidentified. Lane 4, the gangliosides of the tumor in aqueous layer of Folch's partition; Lane 5, ganglioside references. 3 major spots are GA,., GMI, and GD1a, respectively.

Fig. 2. Thin-layer chromatography of glycolipids isolated from the tumor in acetylated forms. Glycolipids in each fraction were acetylated with acetic anhydride:pyridine for 12 hr at room temperature. Solvent system used was dichloroethane:acetone:water (60:40:1, v/v). Visualized with orcinol reagent. Lane 1, acetylated tumor glycolipids in organic layer of Folch's partition. Lane 2, acetylated tumor-neutral glycolipid fraction in aqueous layer of Folch's partition. Lane 3, mobilities of reference glycolipids under the same condition. GA, asialo GMI; Glob, globoside; PG, paragloboside; nHC, norhexaosylceramide; X2, X2 glycolipid purified from human erythrocytes; or, origin; sf, solvent front. Note that GA, and X2 glycolipids, which have almost identical mobility on usual TLC in nonacetylated forms (Fig. 3), are clearly separated in acetylated forms.

Fig. 3. Radioimmunostaining of the tumor glycolipid on TLC plate. Solvent system was chloroform:methanol:water (60:35:8, v/v). After chromatography with this solvent, the TLC plate was soaked in phosphate-buffered saline, pH 7.4, containing 5% bovine serum albumin for 1 hr to avoid nonspecific reaction. Then the plate was incubated with anti-asialo GMI antibody solution (1:1000 diluted) for 1 hr, washed with phosphate-buffered saline containing 1% bovine serum albumin, reacted with rabbit anti-murine immunoglobulin antibody solution (1:1000) for 1 hr, washed, and incubated with 125I-protein A solution for 1 hr. After extensive washings, the plate was dried and subjected to autoradiography. a, Orcinol staining; b, immunostaining with the antibody. Lane 1, neutral glycolipids of type O human erythrocytes in aqueous layer of Folch's partition. Only x2 glycolipid is positively stained. Lane 2, x2 glycolipid is positively stained. Lane 2, x2 glycolipid purified from human erythrocytes showing positive staining. Lane 3, purified globoside, and Lane 4, purified Forssman glycolipid, both showing negative reaction. Lane 5, the tumor glycolipids in organic layer of Folch's partition; Lane 6, the tumor-neutral glycolipids in aqueous layer of Folch's partition. In both Lanes, one of the spots is positively stained, the mobility on TLC of which coincides with that of x2 glycolipid. Lane 7, purified asialo GMI; and Lane 8, purified asialo GM1, both obtained from bovine brain. Asialo GM1 shows strongly positive staining, while asialo GM1 is negative. Glob, globoside; PG, paragloboside; X2, x2 glycolipid; H2, H2 glycolipid; H3, H3 glycolipid; or, origin; sf, solvent front.
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