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Serum β-(1→4)-Galactosyltransferase Activity with Synthetic Low Molecular Weight Acceptor in Human Ovarian Cancer

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Abstract—*A modified procedure was developed for the determination of UDP-galactose: 2-acetamido-2-deoxy-glucopyranoside* β-(1→4)-*galactosyltransferase (GT) in human serum which employed the synthetic substrates* p-*nitrophenyl* 6-0-(2-*acetamido*-2-*deoxy*-β-D-*glucopyranosyl*)-β-D-*galactopyranoside and* p-*nitrophenyl* 6-0-(2-*acetamido*-2-*deoxy*-β-D-*glucopyranosyl*)-α-D-*mannopyranoside as acceptors. The enzyme products were identified by thin layer chromatography with authentic reference compounds, and the galactosyl linkage was characterized by hydrolysis with* β-D-*galactosidase from jack beans. The diagnostic value of this GT for ovarian cancer was tested by measuring the serum enzyme activity in* 28 *ovarian cancer patients with disease,* 20 *ovarian cancer patients with no clinical evidence of disease, and* 22 *healthy females. Although the level of the enzyme activity was significantly higher* ($P < 0.002$) *in the serum of patients with active disease when compared to healthy controls, an appreciable overlap of enzyme activity was found between them. Also, no correlation was found between enzyme activity and tumor size. Differences in methodology and selection of patients makes it difficult to compare results from other reports. However, based on our improved assay procedure, we suggest caution should be exercised in evaluating the merits of GT as a diagnostic marker for ovarian cancer.*

INTRODUCTION

ALTERED levels of total galactosyltransferase (GT) activity have been reported in patients with a number of malignancies [1, 2], but these alterations were neither consistent nor specific. One of the reasons for the discrepancy in these studies is the use of glycoprotein acceptors with ill-defined terminal carbohydrate structures in the assay system that results in the simultaneous determination of several GT activities that are present in the serum [2]. One approach to avoid such inconsistency is to use specific well-defined acceptors for GT assay. In the present communication, we report the availability of the specific synthetic acceptors *p*-nitrophenyl 6-0-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-galactopyranoside (GlcNAc β 1→6 Gal β 1→0φNO_2) and *p*-nitrophenyl 6-0-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-D-mannopyranoside (GlcNAc β 1→6 Man α 1→0φNO_2) for the determination of UDP-galactose: GlcNAc (1→4)-β-D-galactosyltransferase and the activity of GT in ovarian cancer patients using the latter acceptor, GlcNAc β 1→6 Man α 1→0φNO_2 (*p*).

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MATERIALS AND METHODS

Materials

The following compounds were synthesized as described: GlcNAc β 1→6 Man α 1→0φNO_2 (*p*) [3]. Gal β 1→4 GlcNAc β 1→6 Man α 1→0ϕ_2 (*p*) [4]. GlcNAc β 1→6 Gal β 1→0φNO_2 and Gal β 1→4 GlcNAc β 1→6 Gal β 1→0φNO_2 (*p*) [5]. UDP-[^{14}C]-Galactose (337 mCi/mmol) was obtained from New England Nuclear; Boston, MA. Permafluor III and Biosolv were purchased from Packard Instrument Co. and Beckman Instrument Co., respectively. β-D-galactosidase from jack bean was obtained from Sigma Chemical Co. All other materials used were of the highest quality commercially available.

Control sera from healthy females were obtained from voluntary donors to the Blood Bank at RPMI.

Sera from females with ovarian cancer were collected from patients admitted to RPMI's Gynecologic Oncology Department. Seventeen of the 28 patients had serous cystadenocarcinoma of the ovary. The other types of carcinoma of the ovary studied include adeno (5), clear cell (3), mucinous (1), granulosa cell (1), and papillary (1). Fifteen of them had poorly-differentiated carcinoma while 9 had moderate differentiation and 4 had well-differentiated carcinoma. These patients had clinically measurable tumor parameters and were undergoing treatment. Patients with recurrent tumor growth or diseases other than ovarian cancer were excluded. Patients were grouped according to the clinical and/or surgical assessment of the diameter of their largest tumor mass, irrespective of the histology and differentiation of the tumor, namely, small (< 2 cm), moderate (2–10 cm), and large (> 10 cm). Sera was also drawn from treated ovarian cancer patients with no evidence of residual disease either by laparoscopy, laparotomy, or clinical examination. The enzyme was assayed within 24 hr after drawing the blood.

Methods

Enzyme assay. The standard reaction mixture in a final volume of 100 μl contained 0.05 M cacodylate-acetate buffer, pH 7.1, 2.0 mM GlcNAc β 1→6 Man β 1→0φNO_2(*p*), 10 mM $MnCl_2$, 0.5 mM UDP-galactose, 1 mM ATP, 0.02 μCi UDP-[^{14}C]-galactose (337 mCi/mmol) and 10 μl serum. The assay mixture was routinely incubated at 37°C for 1 hr and the reaction was terminated by the addition of an equal volume of cold absolute ethanol. The sample was then centrifuged at 6000 ***g*** for 10 min. The supernatant was spotted on a silica gel TLC plate (0.75 mm thickness) and chromatographed along with the appropriate reference compound, Gal β 1→4 GlcNA β 1→6 Man α 1→0φNO_2 (*p*) or Gal β 1→4 GlcNAc β 1→6 Gal β 1→0φNO_2 (*p*) in ethyl acetate : *n*-propanol : H_2O (3 : 3 : 2, v/v/v) solvent system. After development, the chromatogram was visualized under u.v. light. The spot corresponding to the reference compound was scraped and quantitated in a liquid scintillation spectrometer using 10 ml cocktail containing toluene : Permafluor III : Biosolv (17 : 2 : 1, v/v/v). Control assay tubes contained buffer and water instead of the substrate or enzyme. The enzyme activity is expressed as the percentage of the total radioactivity incorporated into the acceptor per ml of the serum under assay condition.

Product characterization. After thin-layer chromatographic separation of the enzyme product, the spot corresponding to the reference compound was scraped and eluted with water. The eluate was passed through a Bio-Gel P-2 column and the fractions containing the radioactivity were pooled and lyophilized. The lyophilized product was dissolved in 50 μl of 0.05 M glycine–HCl, pH 3.5 and incubated at 37°C for 1 hr with 0.1 units of β-D-galactosidase from jack bean. The liberated [^{14}C]-galactose was quantitated by paper chromatography in *n*-butanol : pyridine : water (6 : 4 : 3, v/v/v) followed by liquid scintillation spectrometry.

RESULTS

GT assay procedure

In our laboratory we have accomplished a chemical synthesis of *p*-nitrophenyl-6-0-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-D-mannopyranoside and *p*-nitrophenyl 6-0-(2-acetamido-2-deoxy-β-D-glucopyranosyl-β-D-galactopyranoside as possible acceptors for GT as well as their expected reaction products Gal β 1→4 GlcNAc β 1→6 Man α 1→0φNO_2 (*p*) and Gal β 1→4 GlcNAc β 1→6 Gal β 1→0φNO_2 (*p*). When the enzyme reaction mixture was chromatographed as described under Materials and Methods, the solvent system employed could resolve the acceptor, enzyme product, UDP-galactose and D-galactose (Fig. 1). The presence of a chromophore, nitrophenyl group, in the substrate and in the reference compound enabled their visualization under u.v. light without any processing. When the spot corresponding to the reference compound on the chromatogram was scraped and the isolated enzyme product treated with β-D-galactosidase from jack bean, all of the radioactivity in the product was recovered as labeled galactose (results not shown). β-D-Galactosidase from jack bean has been reported to specifically cleave the Galβ1→4 GlcNAc linkage [6]. Thus, the human serum contains an enzyme that catalyzes the transfer of galactose from UDP-Gal to GlcNAc β 1→6 Man α 1→0φNO_2 (*p*) and GlcNAc β 1→6 Gal β 1→0φNO_2 (*p*) in a β (1→4) linkage.

β(1→4)-Galactosyltransferase activity as measured by either of the substrates was found to be linear with respect to time from 0 to 3 hr and proportional to the amount of serum protein added up to 4 mg with the percentage of optimal [^{14}C]-galactose incorporation (52%) well within the highest activity observed for cancer patients under the assay condition. The pH optima of the enzyme was 7.2. The apparent K_m for GlcNAc β 1→6 Man α 1→0φNO_2 and GlcNAc β 1→6 Gal α 1→0φNO_2 were 1.2 mM and 1.5 mM, respectively. The assay procedure is reproducible within experimental error of ± 5% as monitored by assaying the same serum sample during the course of the project. The presence of endogenous low molecular weight acceptors or inhibitors in the sera of cancer patients were overruled by mixing experiments wherein the serum

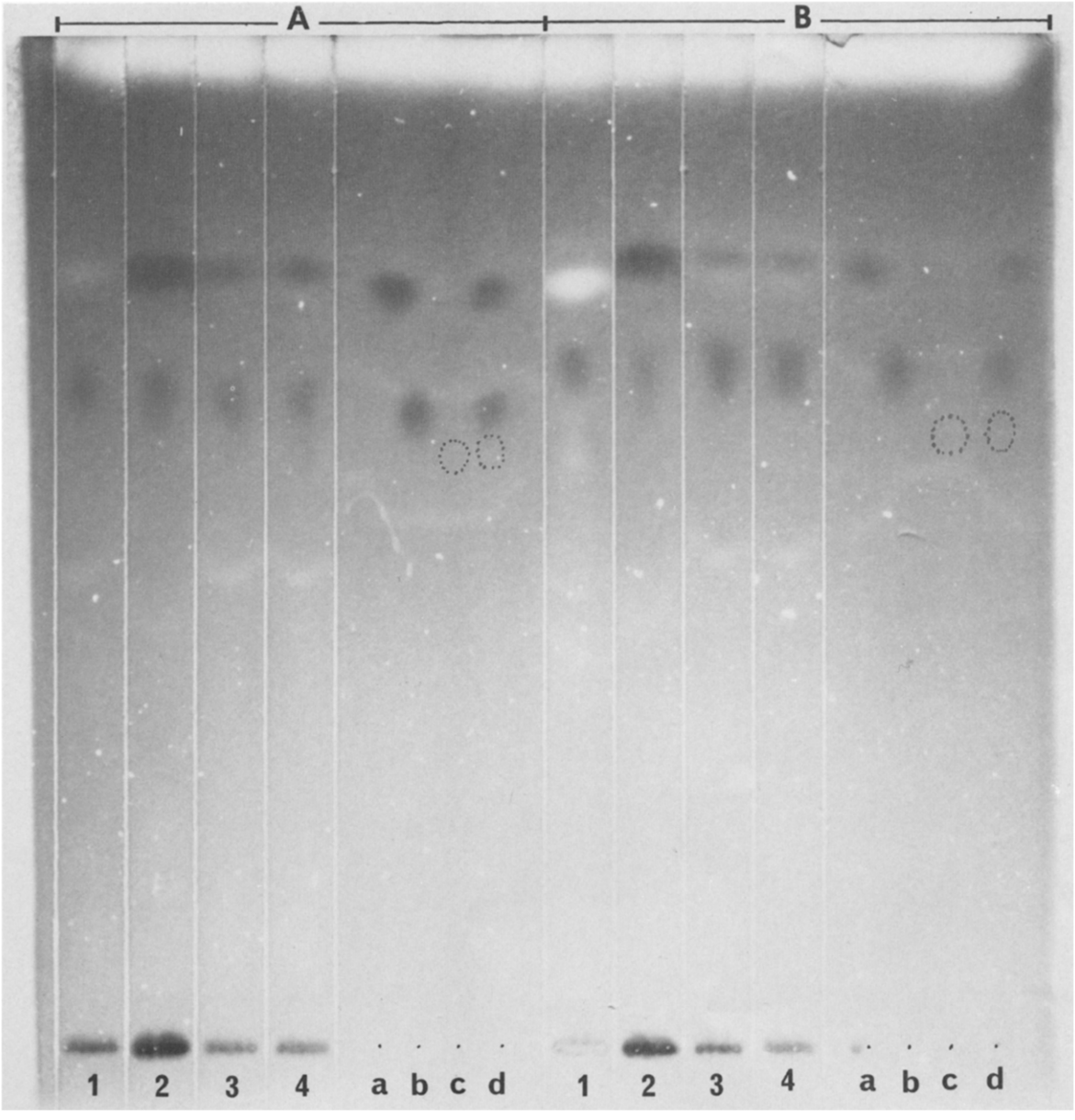

Fig. 1. Representative TLC development in ethyl acetate: n-propanol: H_2O (3 : 3 : 2, v/v/v). Section A represents typical assay using GlcNA β 6→Gal β 1→0ϕNO_2 (p) as the acceptor substrate. Section B represents GlcNAcβ6→Man α1→0ϕNO_2 (p). Scribed channels in each section are as follows: 1—blank (minus enzyme + substrate), 2—control (minus enzyme), 3 and 4—duplicate assays. Spotting in each section indicate following: a—disaccharide acceptor, b—trisaccharide expected product, c—galactose, d—superimposed markers of a, b, and c. For survey, individual patient controls lacking only substrate were included. Galactose was located by later charring plate sprayed with 10% H_2SO_4 in ethanol.

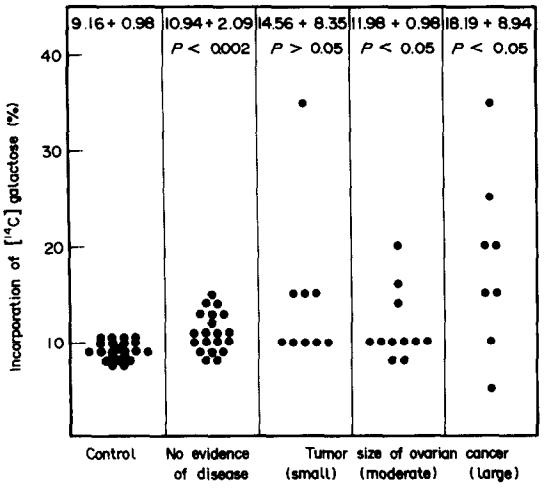

Fig. 2. Serum β-(1→4)-galactosyltransferase activities in ovarian cancer patients, patients cured of the disease, and in healthy females. Mean ± S.D. are given. P *values are in comparison with normal control.* P *value for all the patient with active disease in comparison to healthy control is* (P < 0.002). *Statistics were done in Sperry Univac system using Minitab program.*

of cancer patients, when mixed with normal serum, resulted in additive activity.

GT measurement in ovarian cancer

β(1→4)-Galactosyltransferase activity was measured in sera of ovarian cancer patients using one of the substrates, GlcNAc β 1→6 Man α 1→0φNO_2 (*p*). Sera from patients with known active disease had significantly higher GT activity as compared to healthy control sera ($P < 0.002$) (Fig. 2).

However, when the enzyme level in the sera of ovarian cancer patients cured of the disease was compared to that of the healthy females, a small but statistically significant elevation in the enzyme activity was observed (Fig. 2). Two-fold elevation in GT was also observed in the sera of 4 patients with benign ovarian tumor (data not shown). Further, when the sera of patients were grouped according to their clinical assessment, no correlation was found between β (1→4) GT activity and clinical size of the tumor (Fig. 2). When the tumor size and GT levels were plotted, a correlation coefficient of 0.3 with $P > 0.05$ was observed.

DISCUSSION

GT assay procedure

The diagnostic value of β (1→4) GT measurement for ovarian cancer is often controversial. Although the elevation of this enzyme has been reported in sera of ovarian cancer patients [7–9], recently, Belfield and Pledger [10] questioned the usefulness of the serum GT measurement in the investigation of ovarian cancer. One of the reasons for the controversy is that assay of the enzyme in these studies involved the use of macromolecular acceptors which are undefined with respect to the oligosaccharide chain and are heterogeneous in nature. Such acceptors like asialoagalactofetuin are capable of measuring several isoenzymes of GT [2]. This is more relevant since different isoenzymes of serum GT have been reported to have similar acceptor specificities towards different high molecular weight acceptors of unrelated structures [11]. One approach to avoid such controversy is to use well-defined specific acceptors that closely resemble the naturally-occurring substrates.

In the present communication, we described the use of 2 such acceptors for the measurement of GT. One of the salient features of this modified assay procedure is the simultaneous monitoring of the endogenous glycoprotein acceptor activity, as the protein pellet obtained after adding alcohol can be quantitated by scintillation spectrometry. Levels of some of these endogenous glycoprotein acceptors are known to increase under certain physiological conditions including cancer [12]. Since the solvent system employed in the assay procedure can resolve galactose from the enzyme product (Fig. 1), competing reactions which may interfere with GT assay [7, 13] such as phosphorylase activity that liberates galactose from UDP-Gal, and β-D-galactosidase activity which cleaves galactose from the product formed could also be monitored. However, we did not monitor any of these interfering reactions.

Although GlcNAc has also been used for GT assay [14], the substrate described in the present communication has the advantage of more closely resembling the naturally-occurring substrate for the enzyme, and in addition, the characterization of the enzyme product is much easier.

Clinical evaluation of GT

Although we have solved 1 of the problems associated with GT assay by using a well-defined acceptor for β(1→4) GT measurement, our results do not support the use of this enzyme in the diagnosis of ovarian cancer. For example, an overlap of enzyme activity was found between patients with ovarian cancer and normal controls or patient with no evidence of disease. It is interesting to note that 7 of the 22 patients with no evidence of disease showed elevated levels of GT. Except for 1 patient who had myocardial infarction, the rest did not have any other disease. Such elevation in 46% of the patients cured of ovarian cancer has already been reported [14], but it remains to be seen whether future enzyme levels will decrease after prolonged treatment. Two-fold elevations in GT were also observed in sera of patients with benign ovarian tumors. These observations seem to strengthen the comments of Pohl, 1984 [2] that the changes in GT activity may represent a 'general host response to malignant transformation'. Such unspecific, dis-

ease-induced alterations are unlikely to drop by the removal of the tumor as indicated by significant elevation in the β(1→4)-GT activity in patients with no clinical evidence of disease.

Furthermore, we did not find any correlation between serum GT activity and tumor burden although such correlation was reported in the sera of ovarian cancer patients using high molecular weight acceptors [15]. But it is interesting to note that GT is not elevated in the sera of breast carcinoma patients proportionally to their tumor cell content [16, 17].

Differences in methodology and selection of patients makes it difficult to compare our results with others. However, based on our results, we suggest caution should be exercised when evaluating the merits of GT as a diagnostic marker in ovarian cancer. Nevertheless, the availability of well-defined synthetic acceptors for β(1→4)-galactosyltransferase avoids some of the problems associated with the enzyme assay that may prove valuable in critically evaluating the merits of galactosyltransferase as a diagnostic marker for other cancer.

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