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# GLYCOPEPTIDES FROM MURINE TERATOCARCINOMA CELLS: STRUCTURE OF THE DETERMINANTS RECOGNISED BY *Griffonia simplicifolia* AGGLUTININ I AND BY SERA FROM PATIENTS WITH OVARIAN GERM CELL TUMORS*

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ABSTRACT

High-molecular-weight glycopeptides synthesised by teratocarcinoma OTT6050 bear the binding site for *Griffonia simplicifolia* agglutinin I and are recognised by antibodies in the sera of patients with ovarian germ cell tumors. Digestion of the glycopeptides with endo-β-D-galactosidase C abolished the lectin binding activity and the antigenic activity. Since the product of the enzymic digestion is α-D-Gal-(1→3)-D-Gal, it is concluded that the disaccharide structure is involved in the lectin binding site and the antigenic site.

## INTRODUCTION

Cell-surface carbohydrates undergo marked alterations during differentiation and development[1–4]. The most comprehensive picture of the developmentally regulated alterations in carbohydrates has been obtained in the mouse system. Many monoclonal antibodies and lectins have been found to react with restricted cell populations of the early embryonic cells, and the knowledge is useful for identification and separation of these cells[4]. However, only limited information is available about the biochemical nature of the determinants recognised by the antibodies and lectins. The structure of the determinant is often inferred from the specificity of the antibodies or lectins established by hapten inhibition studies, but elucidation of the structure of the determinant is required. For example, *Lotus tetragonolobus* agglutinin (LTA) binds strongly to the α-Fuc-(1→2)-Gal moiety in hapten inhibition studies[5], whereas most of the fucosyl linkages in LTA receptors

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isolated from teratocarcinoma stem cells were present in β-Gal-(1→4)-[α-Fuc-(1→3)]-GlcNAc[6]. We now report on the α-Gal determinants.

*Griffonia simplicifolia* agglutinin I (GS-I) binds only to extra-embryonic endoderm cells and trophoblasts in early post-implantation mouse embryos[7]. GS-I-$B_4$, an isolectin of GS-I, is strongly inhibited by α-Gal-(1→3)-Gal. Thus, it is possible that the determinant is this disaccharide moiety[8]. However, other possibilities were not excluded, for example, α-Gal-(1→3)[α-Gal-(1→6)]Gal postulated to be present in GS-I-$B_4$ receptors of Ehrlich carcinoma cells[9] may participate in the binding site. In order to study the structure of the determinant, teratocarcinomas, which are tumors composed of cells of all the three germ layers[10], were utilised. The stem cells resemble multipotential cells of early embryos, and the extra-embryonic endoderm cells correspond to these cells in early embryos. Teratocarcinoma cells, especially the stem cells called embryonal carcinoma cells, have large amounts of glycoprotein-bound poly(*N*-acetyl-lactosamine)-type glycan[11,12]. Several cell-surface markers of an oncodevelopmental nature, including the GS-I binding site, are carried by the glycan[2,4]. The culture fluid of *Clostridium perfringens* contains an endo-β-D-galactosidase (endo-β-D-galactosidase C) which releases α-Gal-(1→3)-Gal from several glycoconjugates, including the glycan[13] from teratocarcinoma OTT6050. Therefore, the effect of enzymic treatment on the differentiation marker has been investigated.

We have been interested also in the antigenic determinant recognised by sera from patients with ovarian germ cell tumors[14]. Using the glycan from F9 embryonal carcinoma cells, it was found that ~60% of the patients had a high titre of the antibody reacting with the glycan, whereas normal volunteers had only low reactivity. Subsequent studies revealed that the antigenic determinant is an α-Gal residue, and is similar to the one on Ehrlich carcinoma cells but distinct from blood-group B antigen[15,16]. Therefore, the glycans from teratocarcinoma OTT6050 have been investigated for a similar antigenic determinant.

## EXPERIMENTAL

*Materials.* — Endo-β-D-galactosidase C was isolated[13] from *Clostridium perfringens*. α-D-Galactosidase from coffee bean was purchased from Boehringer Mannheim. D-[$^3$H]Galactose-labelled glycan was prepared from teratocarcinoma OTT6050 cultured for 24 h in the presence of D-[$^3$H]galactose (28 Ci/mmol; 4.2 μCi/mL of the medium) by treatment with 0.2M NaOH and 0.4M $NaBH_4$ for 24 h at 37°, followed by prolonged digestion with pronase, extraction with chloroform–methanol, and column chromatography[17] on Sephadex G-50. Material (Fr. I) eluted near the excluded volume was used as the glycans (Fig. 1). D-[$^3$H]Galactose-labelled glycan from F9 cells (embryoglycan) was prepared as described[12]. GS-I agarose was purchased from E. Y. Laboratories. Sera from patients with ovarian germ cell tumors are those described in previous papers[14,16]; patients M.K., K.O., and H.N. had yolk-sac tumor, embryonal carcinoma, and yolk-sac tumor, respectively.

*Methods.* — A modified Farr assay, hemagglutinin assay, and digestion with α-D-galactosidase from coffee bean were performed as described elsewhere[15]. The labelled glycans from teratocarcinoma OTT6050 were digested with 0.59 mU of endo-β-D-galactosidase C in 50 μL of 0.1M Tris-HCl buffer (pH 7.5) for 24 h at 37°. Under these conditions, no radioactivity was released from poly(*N*-acetyl-lactosamine)-type glycan of F9 cells (embryoglycan) labelled with 2-amino-2-deoxy-D-[$^3$H]glucose or L-[$^3$H]fucose[12]. Affinity chromatography was performed on a column (0.46 × 1.2 cm) of GS-I agarose equilibrated with Dulbecco's phosphate-buffered saline (PBS)[18]. After washing of the column with 4.0 mL of PBS, the absorbed material was eluted with 1 mL of PBS containing 0.2M D-galactose.

## RESULTS

*The antigenic and lectin binding activities of the glycans from teratocarcinoma OTT6050.* — The D-[$^3$H]galactose-labelled glycans from teratocarcinoma OTT6050 were fractionated on Sephadex G-50 (Fig. 1). Three fractions (Fr. I–III) were collected and tested for binding to GS-I agarose and specific elution with 0.2M D-galactose. Most of the labelled glycan (Fr. I) bound to the lectin column (Table I). Lower-molecular-weight fractions bound to the column less efficiently (Table I). The glycans also reacted with sera from certain patients with germ cell tumors (Table II). Sera from normal volunteers gave only slight immunoprecipitation. Thus, the glycan from teratocarcinoma OTT6050 reacted with the sera of patients in a manner similar to that of the glycan from F9 embryonal carcinoma cells (Table II)[14,15].

*Digestion of the large glycan from teratocarcinoma OTT6050 with endo-β-D-*

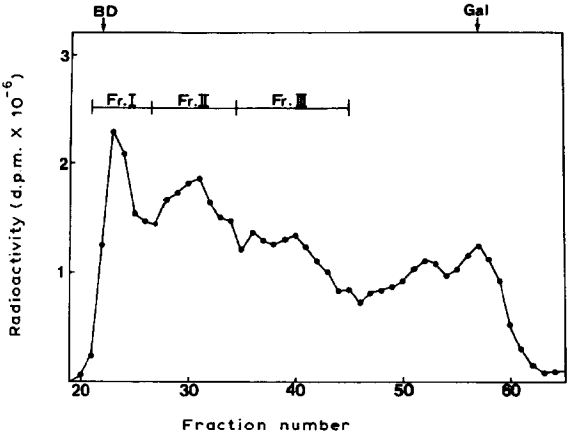


Fig. 1. Fractionation (3.2-mL fractions) of D-[$^3$H]galactose-labelled glycopeptides synthesised by cultured teratocarcinoma OTT6050 on a column (1.8 × 77.5 cm) of Sephadex G-50 (fine) equilibrated and eluted with 0.05M ammonium acetate buffer (pH 6.0). BD and Gal represent the fraction where blue dextran and galactose were eluted, respectively. Glycopeptides: Fr. I (fractions 21–26), Fr. II (fractions 27–34), and Fr. III (fractions 35–45).

TABLE I

EFFECT OF DIGESTION WITH ENDO-β-D-GALACTOSIDASE C ON THE REACTIVITY OF THE GLYCOPEPTIDES FROM TERATOCARCINOMA OTT6050 TO GS-I AGAROSE

| *Glycopeptides*[a] | *Binding (%)*[b] | |
|---|---|---|
| | *Before digestion* | *After digestion* |
| Fr I | 84.5 | 0.8 |
| Fr II | 44.7 | 0.1 |
| Fr III | 16.6 | 0.2 |

[a]Fractionated according to Fig. 1. [b]Per cent of D-[$^3$H]galactose label bound to GS-I agarose and eluted therefrom by 0.2M D-galactose per total radioactivity applied to the column (40,000–50,000 d.p.m.).

TABLE II

COMPARISON OF THE REACTIVITY OF THE SERA OF PATIENTS TO THE GLYCANS FROM TERATOCARCINOMA OTT6050 AND F9 CELLS, AND THEIR HEMAGGLUTINATING ACTIVITY TOWARD RABBIT ERYTHROCYTES

| *Sera from* | *Precipitation (%) of the glycan from* | | *Hemagglutination titer*[a] |
|---|---|---|---|
| Patients | OTT6050[b] | F9[c] | |
| M.K. (yolk-sac tumor) | 28.2 | 22.2 | 64 |
| K.O. (embryonal carcinoma) | 14.8 | 11.3 | 128 |
| Normal volunteers | | | |
| M.O. | 6.4 | 3.4 | 128 |
| T.F. | 7.3 | 6.9 | 64 |
| H.I. | 3.2 | 2.3 | 64 |
| K.K. | 4.7 | 2.9 | 128 |

[a]Maximum dilution to attain hemagglutination of rabbit erythrocytes[15]. [b]D-[$^3$H]Galactose-labelled glycan (Fr. I) (20,000 d.p.m.). [c]D-[$^3$H]Galactose-labelled embryoglycan from F9 cells (20,000 d.p.m.).

TABLE III

EFFECT OF ENDO-β-D-GALACTOSIDASE C DIGESTION OF THE POLY(*N*-ACETYL-LACTOSAMINE) FROM TERATOCARCINOMA OTT6050 ON THE REACTIVITY TO THE SERA OF PATIENTS

| *Sera from patients* | *Precipitation (%)*[a] | |
|---|---|---|
| | *Before digestion* | *After digestion* |
| M.K. | 28.2 | 5.8 |
| K.O. | 17.6 | 4.0 |
| H.N. | 28.3 | 3.8 |

[a]Per cent of D-[$^3$H]galactose-labelled large glycan (Fr. I) (20,000 d.p.m.) precipitated by the modified Farr assay.

*galactosidase C.* — The products of digestion were analysed by gel filtration on columns of Sephadex G-25 (Fig. 2) and of Bio-gel P4 (Fig. 3). With the former column, 27.7% of the label was eluted in the low-molecular-weight region and the remainder in the excluded region. Use of the latter column confirmed that the product released was a disaccharide, and no galactose was detected. Since α-D-galactosidase released 12.3% of the label from the glycan, >80% of the α-Gal was present in the form of an α-D-galactobiose.

After digestion with endo-β-D-galactosidase C, the reactivity of the glycan towards GS-I agarose (Table I) and the sera of patients (Table III) was abolished. Endo-β-D-galactosidase C effects the conversion[13]

α-Gal-(1→3)-β-Gal-(1→4)-GlcNAc→R ⟶ α-Gal-(1→3)-Gal + GlcNAc→R.

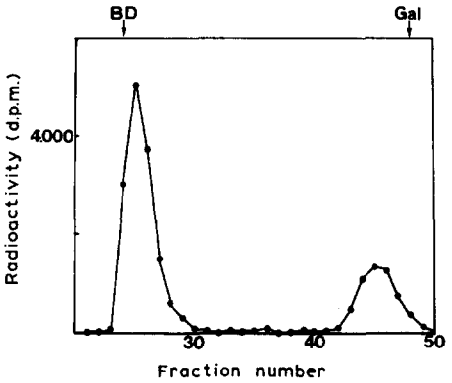


Fig. 2. Analysis of the products of digestion of Fr. I (Fig. 1) with endo-β-D-galactosidase C, which were applied to a column (1.6 × 96.2 cm) of Sephadex G-25 (fine) equilibrated and eluted with 0.05M ammonium acetate buffer (pH 6.0); 3.2-mL fractions were collected. BD and Gal represent the positions where blue dextran and galactose were eluted, respectively.

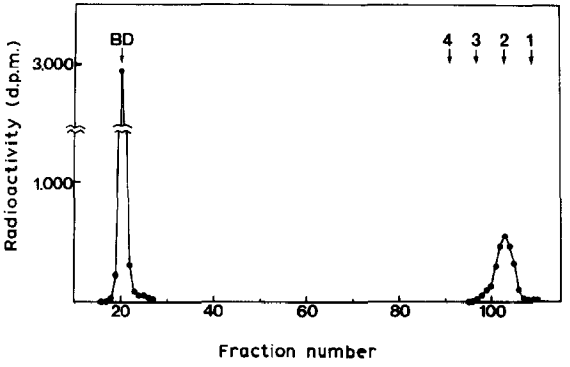


Fig. 3. Analysis of the products of digestion of Fr. I (Fig. 1) with endo-β-D-galactosidase C, which were applied to a column (2.6 × 100 cm) of Bio-gel P4 (>400 mesh) equilibrated and eluted with mM $NaN_3$ at 55°; 4.0-mL fractions were collected. BD represents the position where blue dextran was eluted. Numbers represent the position where dextran oligosaccharides [$(Glc)_n$] were eluted.

Indeed, α-galactobiose released from the glycan from teratocarcinoma OTT6050 grown *in vivo* has the structure α-Gal-(1→3)-Gal[13]. Thus, it is concluded that the structure recognised by the antibody and the lectin involved the α-Gal-(1→3)-Gal.

However, as previously described for the assay using the glycan from F9 cells, the antibody in the patients' sera which reacts with the OTT6050 glycan appears to be different from the heterophilic antibody that agglutinates rabbit erythrocytes; the antigenic component recognised by the latter antibody is α-Gal-(1→3)-β-Gal-(1→4)-β-GlcNAc-(1→3)-β-Gal-(1→4)-Glc-Cer[19]. The agglutinating activity of rabbit erythrocytes towards the sera of patients and normal volunteers is similar (Table II).

## DISCUSSION

Instead of using the glycan from F9 embryonal carcinoma cells in order to elucidate the structure of the epitope involving α-D-galactosidic linkages, the glycan from teratocarcinoma OTT6050, which consists of embryonal carcinoma cells and extra-embryonic endoderm cells, was used for the following reasons. The glycan from F9 embryonal carcinoma cells is less susceptible[20] to endo-β-D-galactosidase C than the glycan from teratocarcinoma OTT6050, probably due to steric hindrance cause by the high degree of branching of the poly(*N*-acetyl-lactosaminyl) chain in the glycan from F9 cells. The structure of the disaccharide released enzymically from teratocarcinoma OTT6050 has been identified as α-Gal-(1→3)-Gal[13].

Furthermore, the use of the teratocarcinoma OTT6050 glycan for elucidation of the GS-I binding site is valid, since the binding site is expressed in extra-embryonic endoderm cells in mouse embryos.

The antigenic epitope recognised by the sera from the patients has been identified as α-Gal-(1→3)-Gal for the glycans from teratocarcinoma OTT6050. The same epitope appears to be associated with the glycan from F9 cells, since it reacts in a manner similar to the glycan from teratocarcinoma OTT6050 with the sera of patients (Table II). Also, oligosaccharides isolated from Ehrlich carcinoma cells inhibited the antibody reacting with F9 glycan[16]. Although poly(*N*-acetyl-lactosamine) synthesised by Ehrlich carcinoma cells was reported to have α-Gal-(1→3)[α-Gal-(1→6)]Gal as a determinant[9], the cell line of the Ehrlich carcinoma cell used here synthesised the α-Gal-(1→3)-Gal structure but not the α-Gal-(1→3)[α-Gal-(1→6)]Gal structure as the major α-galactosyl one[20].

Since the α-Gal-(1→3)-Gal structure has been assigned as a developmentally regulated carbohydrate sequence and as a determinant detectable by antibodies in certain patients with germ cell tumors, glycoconjugates in the tumor with the terminal structure might have promoted the production of the antibodies. Most probably, the α-Gal-(1→3)-Gal sequence is attached to poly(*N*-acetyl-lactosamine) chains by a β-Gal-(1→4)-GlcNAc moiety.

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REFERENCES

1 N. SHARON, *Adv. Immunol.*, 34 (1983) 213–298.
2 T. MURAMATSU, *Cell Differentiation*, 15 (1984) 101–108.
3 T. FEIZI, *Nature (London)*, 314 (1985) 53–57.
4 T. MURAMATSU, *J. Cell Biochem.*, (1987) in press.
5 M. E. A. PEREIRA, E. C. KISAILUS, F. GRUEZO, AND E. A. KABAT, *Arch. Biochem. Biophys.*, 185 (1978) 108–115.
6 Y. KAMADA, Y. ARITA, S. OGATA, H. MURAMATSU, AND T. MURAMATSU, *Eur. J. Biochem.*, 163 (1987) 497–502.
7 T.-C. WU, Y.-J. WAN, AND I. DAMJANOV, *Differentiation*, 24 (1983) 55–59.
8 C. WOOD, E. A. KABAT, L. A. MURPHY, AND I. J. GOLDSTEIN, *Arch. Biochem. Biophys.*, 198 (1979) 1–11.
9 A. E. ECKHARDT AND I. J. GOLDSTEIN, *Biochemistry*, 22 (1983) 5290–5297.
10 G. R. MARTIN, *Science*, 209 (1980) 768–776.
11 T. MURAMATSU, G. GACHELIN, J. F. NICOLAS, H. CONDAMINE, H. JAKOB, AND F. JACOB, *Proc. Natl. Acad. Sci. U.S.A.*, 75 (1978) 2315–2319.
12 H. MURAMATSU, H. ISHIHARA, T. MIYAUCHI, G. GACHELIN, T. FUJISAKI, S. TEJIMA, AND T. MURAMATSU, *J. Biochem. (Tokyo)*, 94 (1983) 799–810.
13 N. FUSHUKU, H. MURAMATSU, M. M. UEZONO, AND T. MURAMATSU, *J. Biol. Chem.*, 262 (1987) 10086–10092.
14 M. KAWATA, K. HIGAKI, S. SEKIYA, H. TAKAMIZAWA, T. MURAMATSU, AND K. OKUMURA, *Clin. Exp. Immunol.*, 51 (1983) 401–406.
15 M. OZAWA, K. HIGAKI, M. KAWATA, S. SEKIYA, H. TAKAMIZAWA, K. OKUMURA, AND T. MURAMATSU, *Biochem. Biophys. Res. Commun.*, 115 (1983) 268–274.
16 M. KAWATA, S. SEKIYA, H. TAKAMIZAWA, T. MURAMATSU, AND K. OKUMURA, *Cancer Res.*, 47 (1987) 2288–2294.
17 H. MURAMATSU, H. HAMADA, S. NOGUCHI, Y. KAMADA, AND T. MURAMATSU, *Dev. Biol.*, 110 (1985) 284–296.
18 R. DULBECCO AND M. VOGT, *J. Exp. Med.*, 99 (1954) 167–182.
19 E. SUZUKI AND M. NAIKI, *J. Biochem. (Tokyo)*, 95 (1984) 103–108.
20 Y. KAMADA, unpublished results.