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Investigating the glycosylation of normal and ovarian cancer haptoglobins using digoxigenin-labelled lectins

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Human haptoglobin (Hp), prepared from 10 normal sera and 10 ovarian cancer sera as well as from 11 ovarian cancer ascitic fluids, was characterized with regard to its reactivities with different lectins. Digoxigenin-labelled lectins [peanut agglutinin (PNA), *Arachis hypogaea*; SNA, *Sambucus nigra*; MAA, *Maackia amurensis*; DSA, *Datura stramonium*; and Con A, concanavalin A] with different carbohydrate specific moieties were used to identify sugar structures in Hp by blotting and by a quantitative assay in multiwell plates [lectin/enzyme-linked immunosorbent assay (ELISA)]. It was found that the lectin blotting was only useful for preliminary investigations, but that the lectin/ELISA gave interesting results that indicated the presence of *N*-linked complex chains. Despite the fact that PNA interacted weakly with desialylated Hp in lectin blotting, no other evidence was obtained to suggest the presence of *O*-linked glycans. Quantitative differences between normal and cancer Hp were observed for Con A, SNA and MAA, but no difference was found in the reaction with DSA. The binding of cancer Hp to Con A and SNA was twice that of normal Hp. Normal serum and ascitic fluid Hp bound similar amounts of MAA, but three times that observed for cancer serum Hp. Our results suggest that normal and ovarian cancer Hp differ in the content of carbohydrate structures containing sialic acid linked $\alpha(2-6)$ or $\alpha(2-3)$ to galactose and the type of oligosaccharide branching.

Keywords: glycosylation, lectins, normal haptoglobin, ovarian cancer serum and ascitic fluid haptoglobins

Introduction

Glycoproteins present in biological fluids consist of mixtures of glycosylation variants, known as glycoforms. In the case of *N*-glycosylated proteins, this means that different forms of oligosaccharide are bound to the same glycosylation site at asparagine (Asn) residues on the polypeptide chain. The *N*-linked structures may exist as one of the following types: 'complex', 'high-mannose' or 'hybrid'. They can also display heterogeneity in these structures, which can be major or minor. Variations including changes in branching, the presence or absence of sialic acids, different types of linkage and the presence of fucose or bisecting *N*-acetylglucosamine have been reported, and are probably the result of the particular metabolic conditions and/or the presence of disease. Recently, much effort has been directed towards discovering whether the quantitative changes in glycoforms of a given glycoprotein are helpful in the diagnosis of disease and/or monitoring the effects of therapy [1–3].

Haptoglobin (Hp) is an acute-phase protein that is present in the plasma of humans and other

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mammals. The concentration of this glycoprotein is increased in the presence of inflammation, malignancy, infection, trauma and tissue damage [4–6]. Hp is synthesized mainly by the liver; however, it has also been reported that it can be produced by ovarian cancer cells [7]. Hp exists as two α- and β-subunits, the β-chain being common in the three main phenotypes: 1-1, 2-1 and 2-2. The carbohydrate on Hp (~20%) is external to the protein and attached to Asn residues on the β-chain at positions 23, 46, 50 and 80. The following sugars are reportedly found in Hp: sialic acid, galactose (Gal), mannose (Man), *N*-acetylglucosamine (GlcNAc) and fucose [5, 8–10].

In the present study, human Hp has been purified from normal sera, ovarian cancer sera and ovarian cancer ascitic fluid. The glycosylation of these Hp preparations has been characterized by using digoxigenin (DIG)-labelled lectins in blotting and in a quantitative assay in multiwell plates (lectin/ELISA).

Materials and methods

Reagents

The 'Glycan Differentiation' kit and Con A (concanavalin A)–DIG conjugate were from Boehringer Mannheim (Mannheim, Germany). This kit contained DIG-labelled lectins (DSA, *Datura stramonium*; SNA, *Sambucus nigra*; MAA, *Maackia amurensis*; PNA, *Arachis hypogaea*), polyclonal sheep anti-DIG Fab fragments conjugated with alkaline phosphatase (AP), 4-nitroblue tetrazolium (NBT) chloride, X-phosphate (5-bromo-4-chloro-3-indolyl-phosphate) and control glycoproteins: carboxypeptidase Y, transferrin, fetuin (Fn) asialofetuin (Afn). The specificities of the lectins used are shown in Figure 1.

Sheep antibodies against mouse IgG (whole molecule) labelled with horseradish peroxidase (A-6782) were from Sigma (St Louis, MO, USA); *p*-nitrophenylphosphate (*p*-NPP) was from Aldrich.

Clinical specimens used

The following specimens were used for Hp isolation:

- Ten blood samples from healthy women, age 28–51, attending for medical follow-up in the Central Lower Silesia Laboratories. The normal sera were chosen on the basis of normal values in a number of laboratory tests (erythrocyte sedimentation, aminotransferase and acid phosphatase activities and haptoglobin and glucose levels).
- Ten blood samples from ovarian cancer patients, age 38–62, attending the 2nd Clinic of Gynaecology and Obstetrics of the Medical Academy in Wroclaw. Ovarian cancers were diagnosed by laparotomy and confirmed by histology. Malignant ovarian tumours included: ovarian endometrioid carcinoma, Figo II; malignant teratoma Figo II; undifferentiated ovarian carcinoma Figo III and IV; undifferentiated carcinoma probably of endometrioid origin, Figo IV. Only three patients were receiving chemotherapy and seven specimens were taken before treatment.
- Ascitic fluids were derived from women suffering from the ovarian cancer, also attending the 2nd Clinic of Gynaecology and Obstetrics of the Medical Academy in Wroclaw.

Unhaemolysed sera, separated by centrifugation, and ascitic fluids samples were stored at −20°C until required for Hp preparation.

Haptoglobin preparation

Hp was prepared from healthy individuals and from ovarian cancer sera by immunoaffinity chromatography on a column containing equal amounts of two anti-Hp monoclonal antibodies coupled to Sepharose 4B, using a method previously described [11]. The Hp–anti-Hp complex was split by 0.1 mol/l acetate buffer, pH 3.8. The fractions were immediately neutralized, pooled, dialysed against phosphate-buffered saline (PBS) and stored at −20°C until used.

Hp was isolated from ovarian cancer ascitic fluid as previously reported [12]. Further purifications being accomplished by eluting from a column of Sephacryl S-300 (Pharmacia, Sweden) with 0.1 mol/l Tris–HCl buffer, pH 8.0, containing 1 mol/l NaCl. After dialysis against distilled water, the Hp solution was lyophilized.

The purity of the Hp preparations was checked by immunoelectrophoresis and by electrophoresis in a 7.5% (w/v) polyacrylamide gel as previously described [11]. Protein concentration was estimated by the Bradford method [13].

Lectin dot blotting

In preliminary experiments with lectins, blotting was performed by either dot blotting or Western blotting. The procedure followed was as described by the manufacturers of the 'Glycan Differentiation' kit. For dot blotting, Hp (0.5–1 μg) or

control glycoproteins were spotted onto dry nitrocellulose (NC) (0.45 μm, BA 85, Schleicher & Schuell, Dassel, Germany) and dried at room temperature. After the blocking and washing procedures, the required amount of DIG-labelled lectin solution (10 μl for each lectin: DIG–PNA, DIG–DSA, DIG–Con A, DIG–SNA and DIG–MAA in 10 ml of buffer) were added and the NC was incubated for 1 h. Then, the NC was rinsed and incubated for 1 h with anti-DIG antibody conjugated with alkaline phosphatase (AP) (anti-DIG–AP). The blot was washed again and the alkaline phosphatase reaction was carried out by incubating the NC, without shaking, in 10 ml of the following freshly prepared solution: 37.5 μl of X-phosphate (50 mg/ml dimethylformamide) and 50 μl of NBT-chloride (75 mg/ml dimethylformamide) in 10 ml of 0.1 mol/l Tris–HCl, pH 9.5, 0.05 mol/l $MgCl_2$, 0.1 mol/l NaCl. For each, lectin-positive and -negative controls supplied by the manufacturers were included on the strip of NC.

Lectin Western blotting

Polyacrylamide gel electrophoresis of Hp preparations in the presence of sodium dodecyl sulfate (SDS), under unreduced conditions, was performed in 7.5% (w/v) gels according to Laemmli [14]. Western blotting based on the procedure of Towbin *et al.* [15] was carried out with some modifications. The transfer of proteins from the gel onto the NC paper was performed using a current of 30 mA at room temperature for 18 h in the transfer buffer. After electrotransfer, the excess binding sites on the NC were blocked by gelatin in PBS containing 0.05% Tween 20 and the DIG–lectin solution was added and incubated with the paper for 1 h at 37°C. The amount of lectin bound to the glycoprotein band was quantified by anti-DIG antibodies conjugated with AP as already described.

Lectin/ELISA

The following procedure was established in multiwell plates to determine quantitatively the binding of Hp to a lectin. Hp (PNA, MAA, 2 μg per well; DSA, 1 μg per well; SNA and Con A, 0.1 μg per well) in 100 μl of Tris-buffered saline (50 mmol/l Tris-HCl and 150 mmol/l NaCl), pH 7.5 (TBS), was coated on a polystyrene microtitre plate (Organon Teknika) and incubated at 37°C for 2 h. The coated plate was washed three times with TBS and blocked with 0.5% gelatin in TBS for 1 h at 37°C and overnight at 4°C. The plate was washed with 1 mmol/l Tris–HCl buffer, pH 7.5, containing 0.05% Tween 20 and 1 mmol/l Ca^{2+}, Mg^{2+}, Mn^{2+}. The plate was incubated with 200 μl of lectin–DIG conjugate (1 μg/ml) in the same buffer as above at 37°C for 1 h. It was then washed and anti-DIG–AP (0.75 U/ml) was added in TBS containing 0.05% Tween 20 and incubated at 37°C for 1 h. The plate was washed again and the AP reaction was carried out by incubating the plate with 1.4 mmol/l *p*-NPP in 1 mol/l diethanolamine–HCl buffer, pH 10.2, for 30 min at 37°C for all the lectins studied except MAA (incubation time 2.5 h). The reaction was stopped with 1 mol/l NaOH and the absorbance was read at 405 nm against a reference wavelength of 492 nm on a Hamilton UVHR 7000 ELISA reader.

Background absorbances (without glycoprotein, but with blocking step, lectin and antibody conjugate) repeated 10 times on different days were as follows: DSA, 0.23 ± 0.03; Con A, 0.53 ± 0.12; SNA, 0.37 ± 0.08; and MAA, 0.19 ± 0.03 AU (mean ± SD). Absorbance values for control without lectin were 0.07–0.09 AU. All absorbances obtained for Hp samples were corrected by subtracting background values. Positive control (1 μg Fn, 1 μg Afn or 0.1 μg Hp pool) were used in the assay. The net absorbances for DSA/Afn, ConA/Hp, SNA/Fn and MAA/Fn were 0.75, 0.51, 0.17 and 0.23 AU respectively. All statistical analyses were carried out using the Student's '*t*'-test.

T-antigen assay

The plate was coated with 0.1 and 1 μg of Hp or 1 μg of glycophorin (positive control) in PBS, then washed four times with PBS containing 0.05% Tween 20 (PBS-T). Thereafter, for desialylation 100 μl of 50 mmol/l sodium acetate buffer, pH 5.0, containing 100 mmol/l NaCl, 0.1% bovine serum albumin (BSA), 0.05% Tween 20, 16 mmol/l $CaCl_2$ and 30 mU/ml neuraminidase from *Clostridium perfringens* was added to each well. The plate was then gently shaken for 3 h at 37°C. After washing with PBS-T containing 2% polyethylene glycol 6000, the plate was incubated with 100 μl of six times diluted hybridoma culture supernatant, containing an antibody against T-antigen (D-Gal-1–3-α-D-GalNAc). Reactivity of this antibody was previously checked with asialo erythrocytes according to Steuden *et al.* [16]. After washing, 200 μl of sheep antibodies against mouse IgG, labelled with peroxidase (1:500), was added for 30 min at 37°C. The plate was washed and 200 μl of substrate solution (0.5% *o*-phenylenediamine in 0.05 mol/l citrate phosphate buffer, pH 5.0, containing 0.06%

H_2O_2) was added. The reaction was stopped after 30 min by addition 50 µl of 12.5% H_2SO_4. The absorbance was measured at 492 nm.

Sugar analysis
Monosaccharides were determined by gas–liquid chromatography (GLC) as alditol acetates [17] after hydrolysis of Hp samples in 4 mol/l trifluoroacetic acid (TFA) at 100°C for 4 h. Perseitol was used as an internal standard. Sugar derivatives were separated using a Hewlett-Packard 5890 instrument, equipped with a mass selective detector. Separations were performed on a capillary column Hp-1 (0.2 mm × 12 m), using the temperature gradient 150–270°C (8°C/min).

Results

Sugar analysis
The results of the sugar analysis of representative Hp preparations are shown in Table 1. As can be seen, the fucose content of Hp from ovarian cancer serum is 50% higher than that of normal serum or ascitic fluid Hp; the Gal content of Hp is also slightly increased in ovarian cancer serum. The most pronounced difference in sugar composition between normal and cancer Hp is in GlcNAc content, which increased from 4.6% (normal serum) to about 6.5% (ovarian cancer).

Lectin blotting
The interaction of different lectins with purified Hp varied; typical examples are given in Figure 1A & B; Con A and SNA gave a strong reaction, DSA a moderate reaction, and the reaction with MAA and PNA was weak. Samples which were treated with lectins after Western blotting were difficult to interpret because the Hp was present as multiple bands. Overall, the intensity of lectin staining was similar for the dot blots and Western blots.

A reaction with PNA was only observed when the Hp preparations were pretreated with neuraminidase, an enzyme which removes sialic acid. Because this result suggested the presence of D-Gal-1–3-α-D-GalNAc, as found in *O*-linked glycans, the Hp preparations were investigated for presence of T antigen and GalNAc. None of the preparations bound to a monoclonal antibody to the T antigen, and no GalNAc was detected in sugar analysis (data not shown). This means that the PNA is not completely specific for β-D-Gal-1–3-αD-GalNAc, and may react weakly with terminal galactoses in *N*-linked glycans [16].

Lectin/ELISA
Figure 2 and Table 2 summarize the results of the lectin/ELISA carried out on the Hp preparations.

1. *Reaction with DSA. Revealing asialoglycoforms.* Hp derived from normal and ovarian cancer samples reacted strongly with DSA, but no differences were found between the normal and pathological samples (Table 2 & Figure 2A).
2. *Reaction with Con A. Binding of biantennary glycans.* Hp from ovarian cancer serum and cancer ascitic fluid reacted twice as strongly with Con A as Hp from normal serum (Table 2 & Figure 2B) and these differences were statistically significant ($P < 0.001$).
3. *Reaction with SNA and MAA. Revealing the (2,6) or (2,3) sialylated* N*-linked sugar chains.* Differences were observed with SNA and MAA. Cancer Hp reacted approximately twice as strongly with SNA as Hp prepared from normal serum ($P < 0.001$). No difference in binding for SNA was found for Hp from ovarian serum and ascitic fluid (Table 2 & Figure 2C). The binding of MAA was low, but the binding to cancer serum Hp was 3.5 times less than the binding to Hp from normal serum and ascitic fluid ($P < 0.001$). The MAA binding to ascitic fluid Hp and normal Hp was not statistically different (Table 2 & Figure 2D).

Table 1. Monosaccharide content of Hp preparations

Hp origin	Fuc	Man	Gal	GlcNAc
Normal serum	0.2 (0.23)	2.9 (3.0)	2.3 (2.4)	4.6 (3.9)
Ovarian cancer serum	0.3 (0.32)	3.1 (3.0)	2.5 (2.4)	6.6 (5.2)
Ovarian cancer ascitic fluid	0.2 (0.25)	2.6 (3.0)	2.0 (2.3)	6.5 (6.1)

Monosaccharide content was determined by GLC and expressed as g/100 g Hp. The values given in parentheses are the number of sugar residues per 3.0 mannose residues. Three preparations were analysed, which were pools of two samples derived from different patients. Values are shown as means of these measurements.

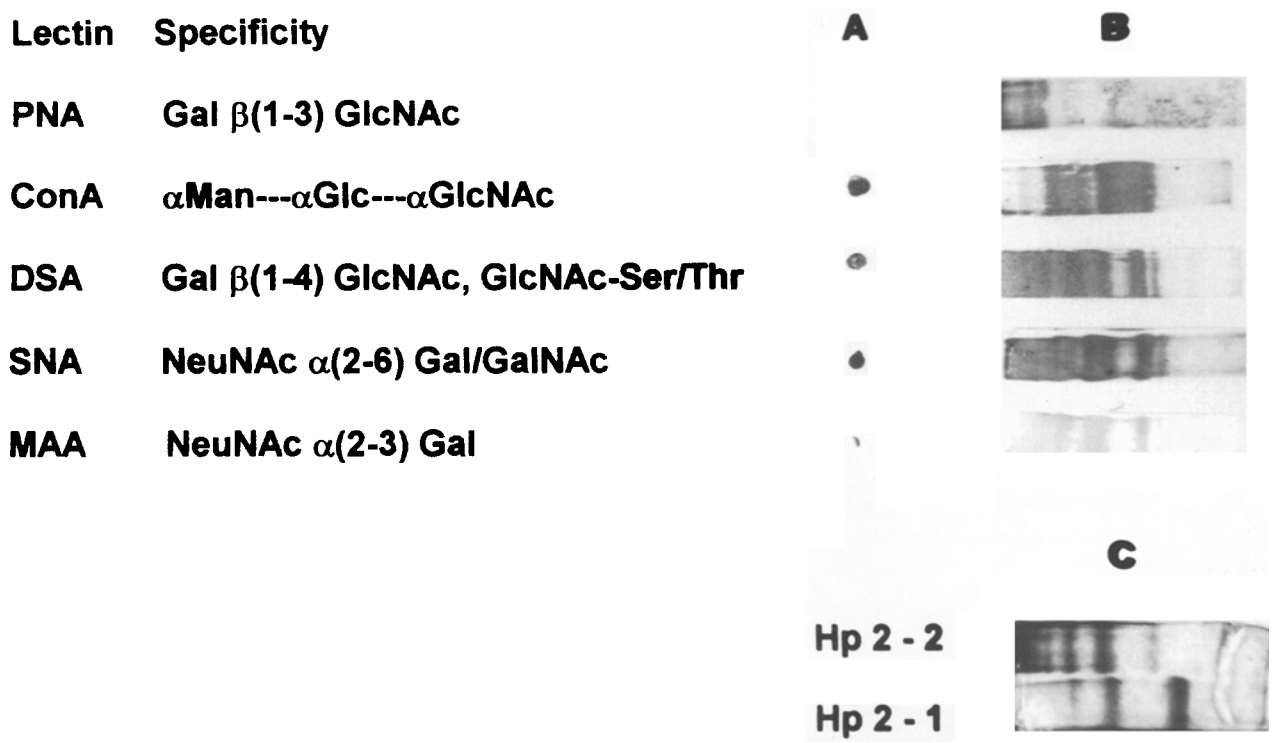

Figure 1. Lectin specificities and preliminary lectin blotting results for Hp. Hp preparations (A, 1 μg; B & C, 20 μg) were either dot blotted (A) or Western blotted (B and C), and treated with either lectins (A and B) or stained with Coomassie blue (C). For PNA, asialo Hp was used and for C Hp preparations 2-1 and 2-2 were stained. For further details see text.

Discussion

Abnormal glycosylation of several circulating proteins in the blood has been detected in various pathological conditions, including inflammation, cancer, autoimmune diseases and alcohol disorders [1–3]. Alterations in the number, type and substitution patterns of the outer sugar residues occur not only in the positive and negative acute-phase proteins (orosomucoid, α_1-antitrypsin, haptoglobin, transferrin), but also in non-acute-phase proteins (α_2-macroglobulin, immunoglobulins) [2,18–20]. These variations in the structure and composition of glycoproteins could have applications in clinical diagnosis [2, 3, 21, 22].

The serum glycoprotein, Hp, exists as a family of molecules (normal, cancer, fetal, polymeric SER (suppressive E-receptor factor), Hp-related protein) that differ in physical, chemical and biological properties and can vary in the degree of glycosylation [5]. There is much evidence to indicate that Hp displays heterogeneity related to different carbohydrate content and oligosaccharide structure [2, 9, 18, 23–27].

In the present paper preparations of Hp derived from normal and ovarian cancer sera, as well as from ovarian cancer ascitic fluids, were characterized with regard to their reactivity with some lectins. Lectins are known to have a high affinity for specific carbohydrate structures; however, this is not absolute, and they can bind to a number of similar carbohydrate structures, albeit with different affinities. In spite of this limitation, some conclusions about carbohydrate structures on Hp can be drawn from our results.

The results indicate that Hp contains *N*-linked oligosaccharide chains, predominantly of the complex type. Similar results have been reported previously by other groups using different techniques [5, 8, 9, 10]. From the known lectin specificities (see Figure 1), it can also be suggested that there are oligosaccharide structures present that contain bi, tri or tetra chains in a lactosamine group with and without sialic acid.

This study has shown that there are quantitative differences in the binding of some lectins to the different Hp preparations (normal and ovarian cancer). The reactivities of MAA and SNA suggested that cancer Hp has more α2–6-linked sialic acid and less α2–3-linked sialic acid than normal Hp. It has been previously shown that α2–3 sialic acid is associated with tri- or tetra-antennary struc-

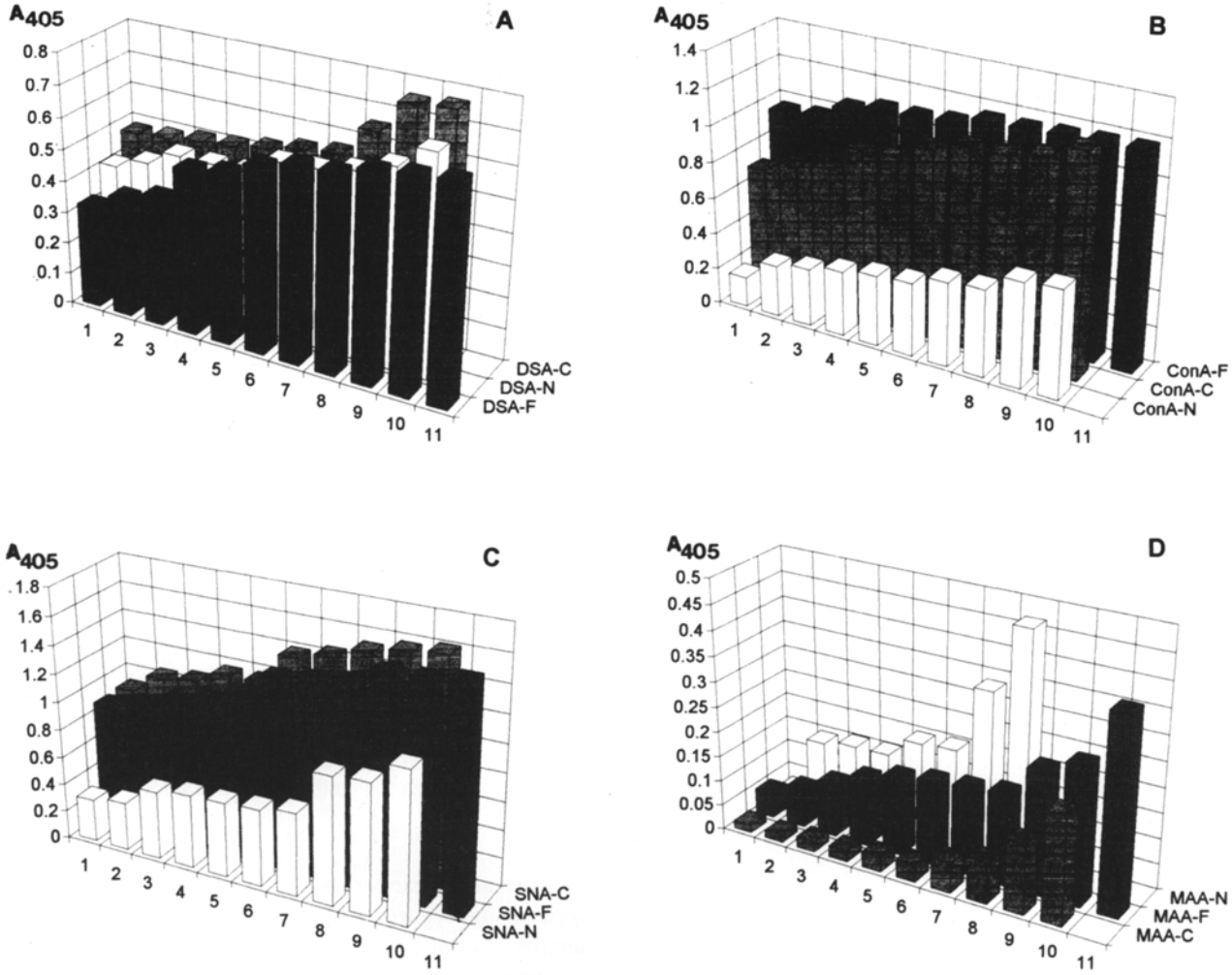

Figure 2. Comparison of Lectin/ELISA results for Hp from normal (N), ovarian cancer (C) and ascitic fluid (F). Reactivity of the Hp preparations was determined as described in the Materials and methods section.

Table 2. A summary of the results obtained for Hp in the lectin/ELISA

Lectin	Source of Hp preparation		
	Normal serum (n = 10)	Ovarian cancer serum (n = 10)	Ovarian cancer ascitic fluid (n = 11)
DSA	0.54 (0.08)	0.58 (0.11)	0.54 (0.05)
ConA	0.40 (0.13)	0.97 (0.17)	1.10 (0.08)
SNA	0.62 (0.26)	1.29 (0.25)	1.26 (0.25)
MAA	0.21 (0.13)	0.06 (0.06)	0.18 (0.09)

Results are expressed as the mean absorbance at 405 nm (SD in parentheses). For each Hp preparation, lectin reactivity was measured nine times and the mean and SD were calculated from the average value of these measurements.

tures [3]. Our finding, therefore, could indicate that cancer Hp has more biantennary chains than normal Hp. This agrees with greater reactivity of the cancer Hp with concanavalin A, a lectin that preferably binds to 2-substituted α-mannopyranosyl residues in the core sequence of N-linked glycans. On the other hand, our analysis of the monosaccharide content of Hp indicated a higher GlcNAc content in cancer Hp. This finding confirms that reported by Thompson *et al.* [9] and Dargan *et al.* [10]. This could suggest the presence of bisecting GlcNAc residues. This change has been shown to be present in the N-linked glycans of several glycoproteins in cancer, *e.g.* transferrin, γ-glutamyltranspeptidase [28]. However, increasing bisecting GlcNAc would tend to reduce the interactions of cancer Hp with Con A [2]. This was not

observed. Further investigation is required to clarify this situation.

Thompson *et al.* [9] and Dargan *et al.* [10] did not find any differences in the total sialic acid content of Hp derived from healthy women and from patients with ovarian cancer with progressive disease but this sugar was significantly lower in the sera of patients in remission. They also found a small increase in the galactose content in ovarian cancer Hp preparations. We did not observe a difference in the reactivities of normal and cancer Hps for DSA, a lectin that gives a positive reaction with disaccharide galactose-β (1–4)-*N*-acetylglucosamine. This lectin can only react with oligosaccharides from which the terminal sialic acid residues have been removed, leaving exposed terminal galactoses [29].

Cancer Hp from serum and ascitic fluid seemed to have very similar lectin reactivity, except with MAA. It is unclear if this difference is caused by different metabolism of glycoproteins in ascitic fluid or the different method (ion exchange and molecular sieving versus immunoaffinity chromatography) used for the preparation of Hp from ascitic fluid.

Thompson *et al.* [9], Dargan *et al.* [10] and Thompson & Turner [30] have shown that in cancer an abnormally fucosylated form of Hp occurs. The fucose content of Hp is low when the cancer is benign or in remission, and much higher when the disease is progressive. We believe that quantitative estimations of the fucosylation, number of glycan antennae, and the extent of asialo forms in Hp could lead to better understanding of its metabolism in cancer, and to clinical methods of better diagnostic and prognostic value.

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