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A two-site lectinoenzymatic assay for determination of tumour marker glycoproteins in rectal secretions

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A method is described for a titre-tray based two-site lectinoenzymatic assay of glycoproteins. WGA lectin, reacting with the core-part of glycans, was combined with lectins PNA and DBA, the latter two reacting with terminal parts of glycans. A standard curve was obtained with bovine submaxillary gland asialomucin, and measurements of human rectal secretion were calibrated against this curve. The assay showed an intra-assay reproducibility of 2.4–7.5%, and inter-assay reproducibility of 3.9–20.8%. Recovery tests showed a linearity close to predicted values. The selected standard was ideal as inhibition of lectin binding by monosaccharides showed similar inhibition profiles for human rectal secretion and for asialomucin standard. Neuraminidase treatment dramatically increased the PNA binding to human rectal secretion immobilized on WGA. Western blotting of human rectal secretion demonstrated a large range of lectin-reactive glycoproteins, the main fraction reacting with all lectins being approximately 250 kDa. The assay described is well suited for studies of the glycan part of tumour marker glycoproteins, and changes occurring in these. It has a high sensitivity by ignoring that the glycans may be present on different molecules. Examination of rectal secretions from various cancer patients showed significantly increased PNA binding, as well as an increased PNA/DBA binding ratio, in patients with colorectal cancer ($p<3\times10^{-3}$) and, unexpectedly, in patients with other cancers ($p<5\times10^{-3}$).

***Keywords*: Lectin-assay, mucins, rectal secretion, cancer detection, glycoproteins**

***Abbreviations*: HRS, human rectal secretion; PNA, peanut agglutinin; DBA, dolichos biflorus agglutinin; WGA, wheat germ agglutinin; BSA, bovine serum albumin; ELLSA, enzyme linked lectino-solid-phase assay; HRP, horseradish peroxidase; HRS: human rectal secretion; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; prot., protein; kDa, kilodalton; OPD, Ortho-phenylen-diamine; SA, Sialic acid; Gal, Galactose; GlcNAc, *N*-acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine; Fuc, fucose; Man, mannose; Glc, glucose**

Introduction

The carbohydrate portions of glycoconjugates, vary during development and in various disease states like cancer and inflammatory diseases [reviews, 1, 2]. Some of the molecules carrying these carbohydrates have been characterized as mucins in diseases like ovarian carcinomas [3], pancreatic and colorectal carcinomas [4], and various other malignancies [5].

Mucins have recently caught attention as they are not only a secretory product but also integral cell-membrane proteins that mediate cell adhesion. In this adhesion, the carbohydrate chains are essential for the adhesive function of the mucin [6]. In the rectum, WGA binding carbohydrate structures are present in both normal and malignant mucosa [7]. Other structures, capable of binding PNA [8] and UEA_1 [9] lectins, or expressing Le^b, Le^x, Le^y [10], Tn [11], TF [12] and ABH antigens [13], are mostly restricted to malignant tissue and surrounding transitional mucosa and do not occur in normal rectal mucosa. A third subset of structures, to which DBA binding structures belong [7], behave in an opposite way as they are present in normal mucosa but reduced or absent in malignant mucosa.

Mucins and most other glycoproteins have several glycans per molecule, enabling simultaneous binding of two different glycan detecting ligands, lectins or antibodies, to one single glycoprotein molecule.

In the present study, combinations of the lectins, WGA, PNA, and DBA were used in enzyme linked lectino-solid-phase assays (ELLSA), to quantitate specific changes in the glycan part of glycoproteins, obtained from human rectal mucosa. WGA is generally bound to the constant core-region of glycans present on most glycoproteins [14], whereas PNA [15] and DBA [16] show a more restricted binding to terminal oligosaccharide sequences.

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In order to assay different glycoproteins, a standard glycoprotein was chosen which reacted positive in all assay combinations. Standard curves obtained with this glycoprotein served as a reference against which all examined glycoproteins were calibrated. The ligand binding to a certain sample was then expressed as the corresponding amount of standard glycoprotein giving the same amount of binding. The various glycoproteins reacting in the assay were characterized by SDS-PAGE, and Western blotting with lectins.

The aim of this study was to establish an assay that could be used to detect carcinomas by the use of rectal secretion as target, and altered glycosylation as tumour associated variable. Therefore, the assay was applied to samples of rectal secretion from healthy controls and patients with colorectal cancer as well as other cancers. The assay showed a highly significant cancer associated increased PNA and reduced DBA binding to glycoproteins immobilized on WGA.

Materials and methods

Materials

Ninety-six micro-well titre trays, Maxisorp, Nunc, Roskilde, Denmark; WGA (Wheat Germ Agglutinin, binds to $(GlcNAc)_2$-R [14]), PNA (Peanut Agglutinin, binds to Galβ1-3GalNAc-R [15]), DBA (Dolichos Biflorus Agglutinin, binds to GalNAcβ-R [16]), all lectins with and without conjugation with horseradish peroxidase (HRP); bovine submaxillary mucin, asialofetuin, asialomucin, asialoglycophorin, D+ galactose, D+ glucose, p-L-glucose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, D+ mannose, Neuraminidase Type X, 3-amino-9-ethylcarbazole, and mercaptoethanol were from Sigma Chem. Co. (St Louis, MO, USA). Protein determination kit was from BioRad, Richmond Ca., USA, Bovine serum albumin, Triton X-100, and Tween 20 were from Merck, Darmstadt, FRG. Cellulosenitrate, 0.45 µm, from Schleicher and Schuell (Dassel, Germany). 1,2-phenylendiamine-dihydrochlodie (OPD) tablets 2 mg, Dakopatts (Glostrup, Denmark). Gelatine G was from Grindsted Products (Brabrand, Denmark).

Methods

Human rectal secretion

Sampled from individuals with rectal carcinomas and from healthy individuals without any history of colorectal diseases. Secretion from rectal mucosa was collected by a piece of cloth that was inserted into the gut lumen through the rectoscope during rectoscopic examination. After this, the piece of cloth was stored frozen at $-20\,^{\circ}C$. The frozen samples were thawed, and 1 ml 0.15 M NaCl containing 1% Triton X-100 was used to wash the secretion out of the cloth. All samples were centrifuged at $800 \times \mathbf{g}$ for 30 min at room temperature, whereafter the supernatant was used for the assay. Protein content of the supernatant was measured in a BioRad assay. A typical protein content of the supernatant was in the range 1 mg ml^{-1} to 20 mg ml^{-1}.

Enzyme linked lectin solid-phase assay (ELLSA)

The following assay conditions were kept constant in all assays, coating buffer: 0.1 mol l^{-1} $NaHCO_3$ (pH 9.8). Washing solution, and solution for dilution of samples, ligands and blocking reagents: PBS (0.5 M NaCl, 0.0075 M Na_2HPO_4, $2H_2O$, 0.0025 M NaH_2PO_4, H_2O), (pH 7.2) containing 0.1% tween 20. Enzyme substrate: 8 mg OPD in 12 ml 0.1 M citric acid phosphate buffer (pH 5.0). Five µl 30% H_2O_2 were added 5 min before use. Scanning: all wells were read in a Tim 200 immunoreader (Teknunc, Roskilde, Denmark), at 492 nm, with subtraction of 620 nm.

Sequence of steps and their variation in the ELLSA procedure

(1) Coating of wells with 100 µl capture lectin overnight at 4 °C. The lectins (PNA, WGA) were tested in concentrations ranging from 1 µg ml^{-1} to 64 µg ml^{-1}. Incubation with inhibiting monosaccharide abolished binding of secretion to the capture lectin.
(2) Washing three times in washing buffer.
(3) Blocking with blocking buffer. Two hundred µl of the following solutions were tested: 2% BSA in washing buffer, or 2% Gelatine G in washing buffer, for 10 min at 20 °C. 2% BSA proved to be superior, and was used as standard blocking solution. Incubation time was tested from 5 to 60 min, and 10 min proved to be optimal.
(4) Washing once in washing buffer.
(5) Incubation with 100 µl sample, standard or control solution. Incubation times between 30 min and 18 h were tested, and 2 h proved to be optimal.
(6) Washing once in washing buffer.
(7) Incubation with 100 µl labelled lectin for 1 h at 20 °C. The following lectins were tested WGA-HRP, PNA-HRP, DBA-HRP, in concentration ranges between 0.02 µg ml^{-1} and 2 µg ml^{-1}. The following concentrations proved optimal: WGA-HRP, 0.05 µg ml^{-1}; PNA-HRP, 0.33 µg ml^{-1}; DBA-HRP, 0.2 µg ml^{-1}. The lectins were diluted in washing buffer containing Mn++ ions, and the pH of the buffer was varied between 6.0 and 10.0 to assure optimal specific binding. The incubation time was 60 min. A pH of 7.6 was optimal for all ligands, and was used routinely.
(8) Washing once in washing buffer.
(9) One hundred µl of enzyme substrate for 15 min at room temp.
(10) The colour development was stopped by addition of 150 µl 1 M H_2SO_4 to each well.
(11) Scanning of the wells at 492 nm.
(12) Calculating the results from the standard curve, in a semilogarithmic plot using computerprogram LOTUS 123, from Lotus Development Corporation, Cambridge, MA, USA. The background absorbance without sample (DBA: 1.2%, PNA: 4.5%, WGA: 7.5%) was subtracted.

Neuraminidase treatment of human rectal secretion

Two hundred μg (protein) TritonX-100 solubilized human rectal secretion was dialysed for 24 h in Spectrapor no. 3 against H_2O, followed by dialysis for 24 h at 4 °C against 10 mM $CaCl_2$, 150 mM NaCl (pH 5.5). The rectal secretion was then incubated with 0.5 U ml^{-1} neuraminidase for 4 h at 37 °C. Control rectal secretion from the same individual was incubated without enzyme. After neuraminidase treatment, the rectal secretion was dialysed against water, then against washing solution (vide supra), in the same way as described above. To measure the effect of neuraminidase, an equal amount of enzyme treated and control rectal secretion mucin was assayed with the WGA-PNA.HRP and WGA-DBA.HRP assays.

SDS-PAGE and Western blotting

Rectal secretion samples were freeze dried and boiled in 4% (w/v) SDS, 10% (v/v) mercaptoethanol for 3 min, separated in a 4–15% gel by the use of a Phast System from Pharmacia LKB Biotechnology, Uppsala, Sweden. The gels were blotted onto nitrocellulose in the Phast system, blocked in 1% tween 20 in buffer A (50 mM Tris, 150 mM NaCl, pH 7.4) for 15 min, incubated at 4 °C for 1 h with peroxidase labelled lectins diluted in buffer A, washed in 0.05% tween 20 in buffer A, and reacted with 3-amino-9-ethyl-carbazole. India ink staining of lanes served as control of the blotting efficiency. Neuraminidase treatment of blots was done with 0.1 U ml^{-1} neuraminidase in 0.9% NaCl, 0.01 M $CaCl_2$ (pH 5.5) for 30 min at 37 °C, prior to incubation with lectin.

Results

Standard curves

The standard curves obtained in the WGA-PNA.HRP, WGA-DBA.HRP, and PNA-WGA.HRP assays are shown in Figure. 1A–C. Bovine submaxillary gland asialomucin served as standard in all assays. Asialofetuin and asialoglycophorin were tested but showed only binding in some of the assays. The optimal coating concentration of the immobilized capture lectin was 5 μg ml^{-1}. The standard asialomucin was assayed in the ranges 0.1–10.0 μg prot. ml^{-1} (WGA-PNA.HRP), 0.02–10 μg prot. ml^{-1} (WGA-DBA.HRP), and 0.01–100 μg prot. ml^{-1} (PNA-WGA.HRP). Human rectal secretion was assayed in the ranges 100–1000 μg prot. ml^{-1} (WGA-PNA.HRP and WGA-DBA.HRP), and 10–500 μg prot. ml^{-1} (PNA-WGA.HRP) (Figure 1A–C). Based on the titration curves of rectal secretion it was decided to calibrate the absorbance from individual samples at a concentration of 250 μg prot. ml^{-1} against the standard curve, and use the standard asialomucin concentration giving that absorbance as a single point result.

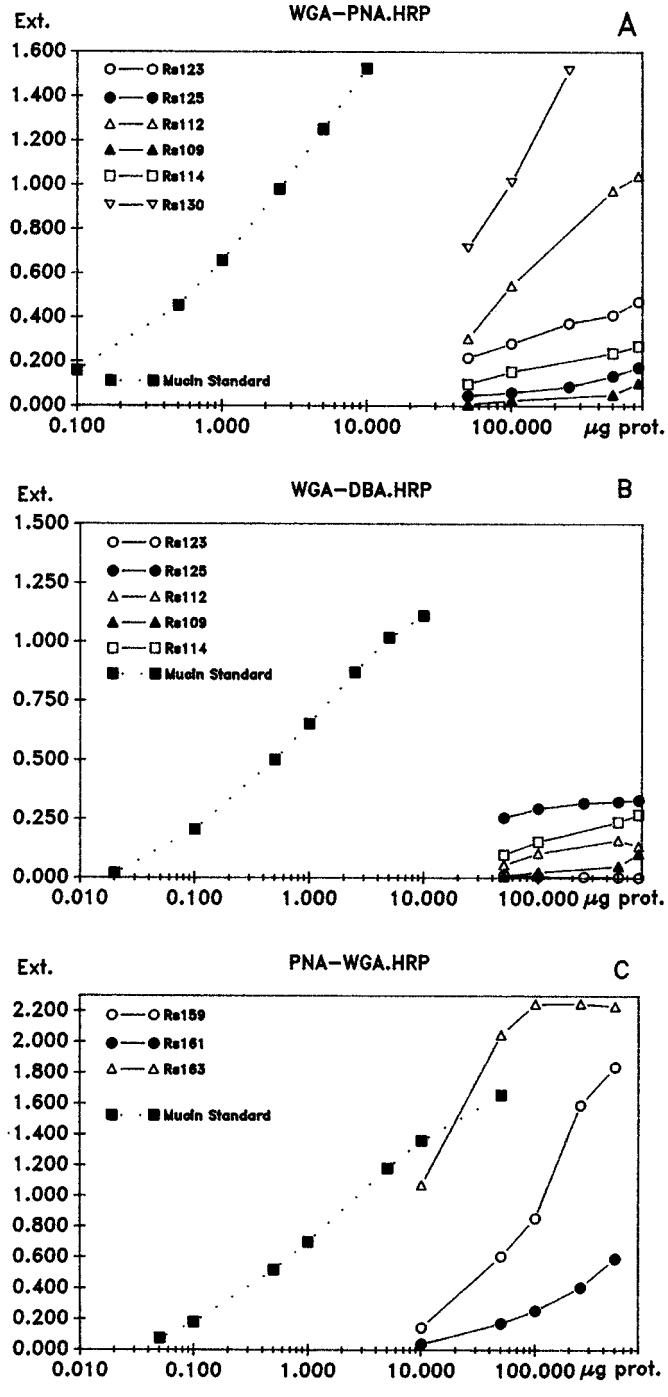

Figure 1. Titration of bovine submaxillary asialomucin standard and human rectal secretion (Rs) from patients with normal rectum (filled symbols) and rectal carcinomas (open symbols). (A) WGA as captive lectin, PNA conjugated with HRP as labelling lectin. (B) WGA as captive lectin, DBA-HRP as labelling lectin. (C) PNA as captive lectin, WGA-HRP as labelling lectin.

Validation of the assay

To determine intra-assay reproducibility, each concentration was assayed multiple times, in duplicate (Table 1). The coefficients of variation ranged from 2.4 to 7.5%. The inter-assay reproducibility was tested by successive assays of a series of concentrations, in duplicate, during 1 week (Table 2). The coefficients of variation ranged in this case

Table 1. Intra-assay variation of three different lectin based assays, used for determination of lectin binding to human rectal secretion.

Assay	n	*µg, mean*	*SD*	*CV(%)*
WGA-PNA.HRP	33	0.31	0.0237	7.5
–	20	0.70	0.0316	4.5
WGA-DBA.HRP	31	0.16	0.0127	2.4
–	18	0.79	0.0293	3.7
PNA-WGA.HRP	22	0.59	0.031	5.2
–	27	1.21	0.043	3.6

Human rectal secretion was assayed at a concentration of 250 µg ml^{-1} prot. per well. Secretions from six individuals were examined to cover different levels of lectin binding. *n*, number of repeated measurements; SD, standard deviation; CV, coefficient of variation.

Table 2. Inter-assay variation of three different lectin based assays, used for determination of lectin binding to human rectal secretion.

Assay	n	*µg, mean*	*SD*	*CV(%)*
WGA-PNA.HRP	4	0.26	0.022	8.6
–	4	0.42	0.026	6.2
–	5	0.60	0.047	7.9
–	4	1.77	0.296	16.8
WGA-DBA.HRP	4	0.04	0.0082	20.4
–	4	0.60	0.057	9.6
–	4	1.77	0.165	9.3
–	4	3.4	0.22	6.4
PNA-WGA.HRP	5	0.16	0.024	14.7
–	5	0.27	0.025	9.4
–	5	0.67	0.026	3.9
–	5	1.94	0.270	13.9
–	5	2.64	0.550	20.8

See footnotes to Table 1.

from 3.9 to 20.8%. Recoveries were evaluated in two ways. By adding dilutions of rectal secretion from one patient to a standard amount of rectal secretion from another patient (data not shown) and assaying. The recovery ranged in this way from 88 to 109%. Another recovery test performed with the WGA-PNA.HRP and PNA-WGA.HRP assays was the mixing of a human rectal secretion sample, giving a low value, to a sample giving a higher value, followed by assessment of the linearity (Figure 2A–C). The ideal curve for the mixture would be exact in the middle between the curves. The curves obtained were very close to this ideal curve (Figure 2). Storage of samples overnight at 4 °C, or storage for weeks at minus 80 °C before assaying did not alter the results (data not shown).

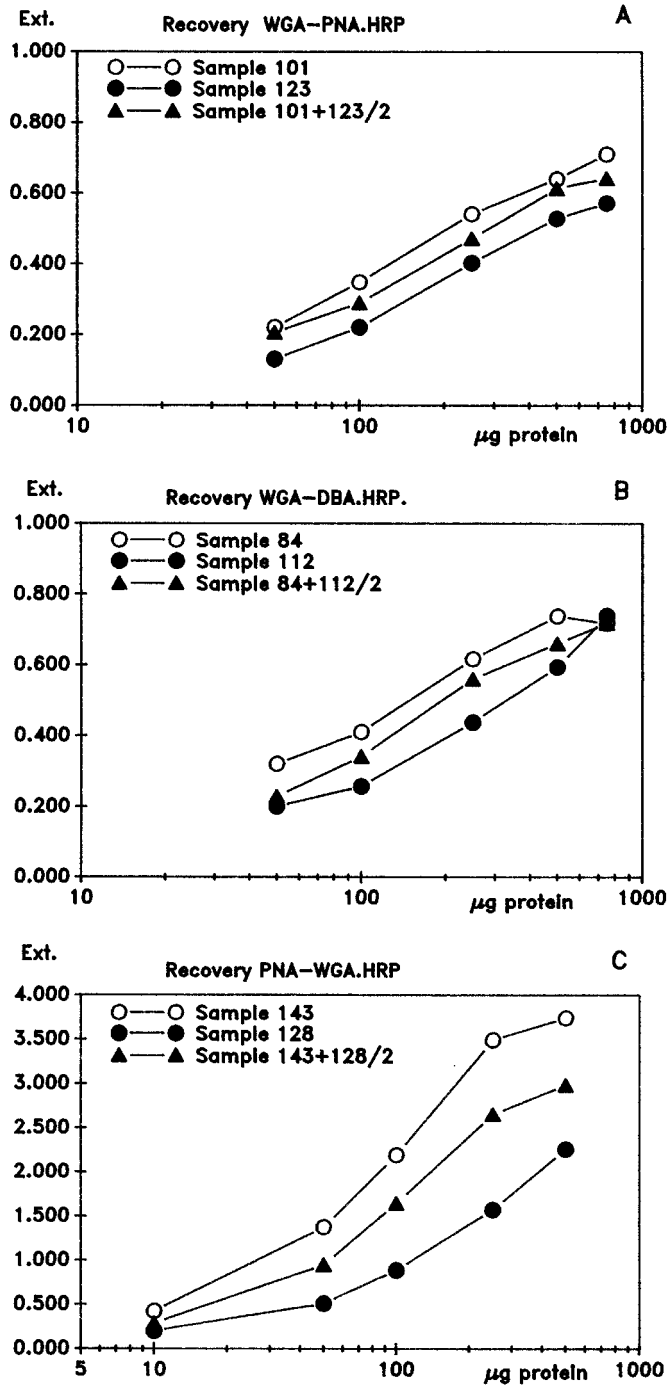

Figure 2. Recovery experiments based on titration of samples giving high and low values, and mixtures of these. All samples originated from patients with rectal carcinomas in (A) and (C), and from normal rectum in (B). (A) WGA as captive lectin, PNA conjugated with HRP as labelling lectin. (B) WGA as captive lectin, DBA-HRP as labelling lectin. (C) PNA as captive lectin, WGA-HRP as labelling lectin.

Monosaccharide inhibition studies

The WGA-PNA.HRP assay of a human rectal secretion was completely inhibited by preincubation of the PNA.HRP lectin with 0.01 M galactose (Figure 3A). Other monosaccharides showed less or no inhibition. The asialomucin standard showed an essentially similar inhibition profile as human rectal secretion (Figure 3B). In case of the WGA-DBA.HRP

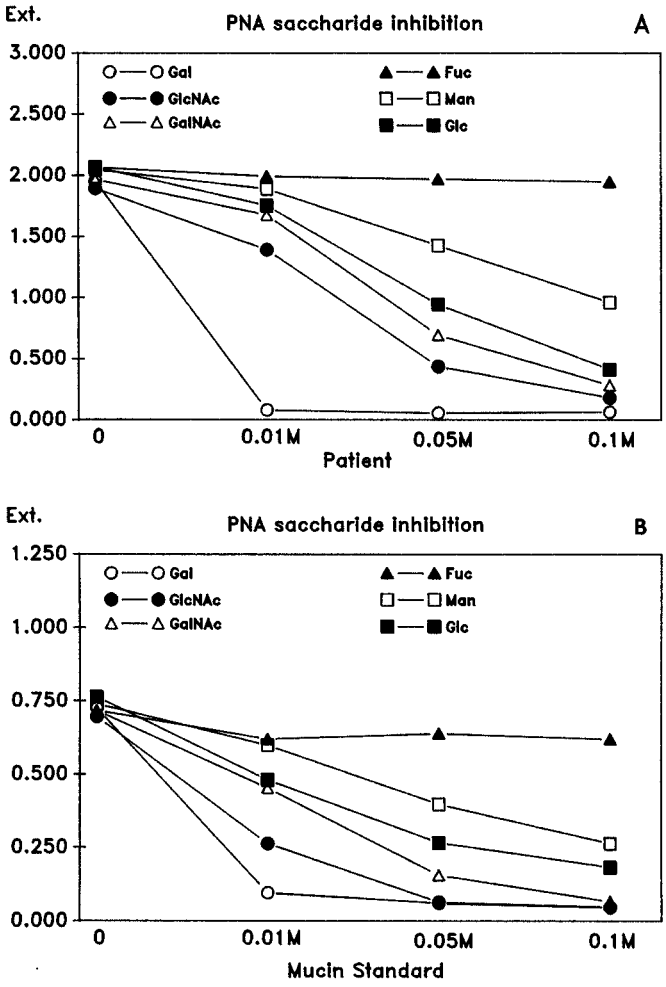

Figure 3. Monosaccharide inhibition of PNA-HRP binding to glycoproteins captured by WGA. (A) Sample from patient with rectal carcinoma. (B) Bovine submaxillary asialomucin standard.

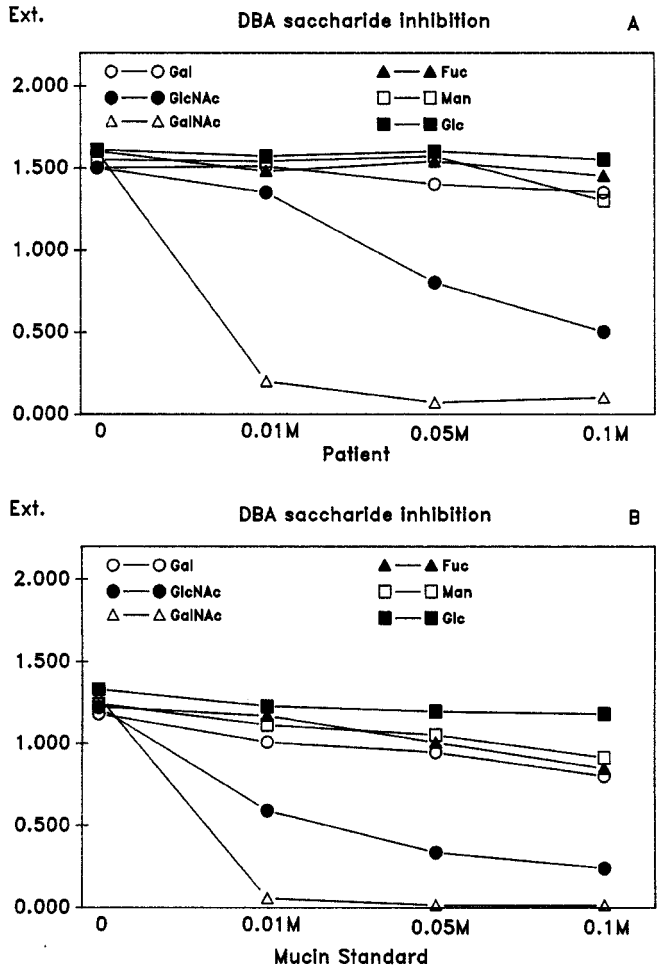

Figure 4. Monosaccharide inhibition of DBA-HRP binding to glycoproteins captured by WGA. (A) Sample from patient with normal rectum. (B) Bovine submaxillary asialomucin standard.

assay both rectal secretion (Figure 4A) and standard asialomucin (Figure 4B) was inhibited by 0.01 M *N*-acetylgalactosamine, and showed similar inhibition profiles. The PNA-WGA.HRP assay was inhibited by *N*-acetylglucosamine, and also in this case showed the human rectal secretion and standard mucin identical inhibition profiles (data not shown). In conclusion, the asialomucin standard seemed to be an ideal reference for all three assays.

Neuraminidase treatment

Human rectal secretion, which was negative or weakly positive in the WGA-PNA.HRP assay, was treated with neuraminidase. This resulted in a strong increase in extinction (data not shown). Two samples were increased less than the others, but still showed an increase by a factor 2.5 and 5. The WGA-DBA.HRP assay was insensitive to neuraminidase treatment.

Partial characterization of the glycoproteins

This involved SDS-PAGE and was run with human rectal secretion and blotted onto nitrocellulose, followed by staining with peroxidase labelled lectins identical to those used in the two-site assay. All samples bound WGA to a large extent, mostly in the form of mucin-smears (Figure 5), extending from the low to the high molecular weight area. PNA also bound to a mucin smear and, in addition, showed a preference for molecules at a molecular mass of approximately 250 kDa and 60 kDa. After neuraminidase treatment of the blots, PNA showed a highly increased staining of mucin smears. DBA mostly reacted with the 250 kDa fraction that also bound PNA, but also showed a mucin smear in the most positive case. Neuraminidase treatment of the blots had no significant effect on the DBA binding. The staining of the blots showed a good correlation to the extinction found with the same samples in the titre tray assay (footnote to

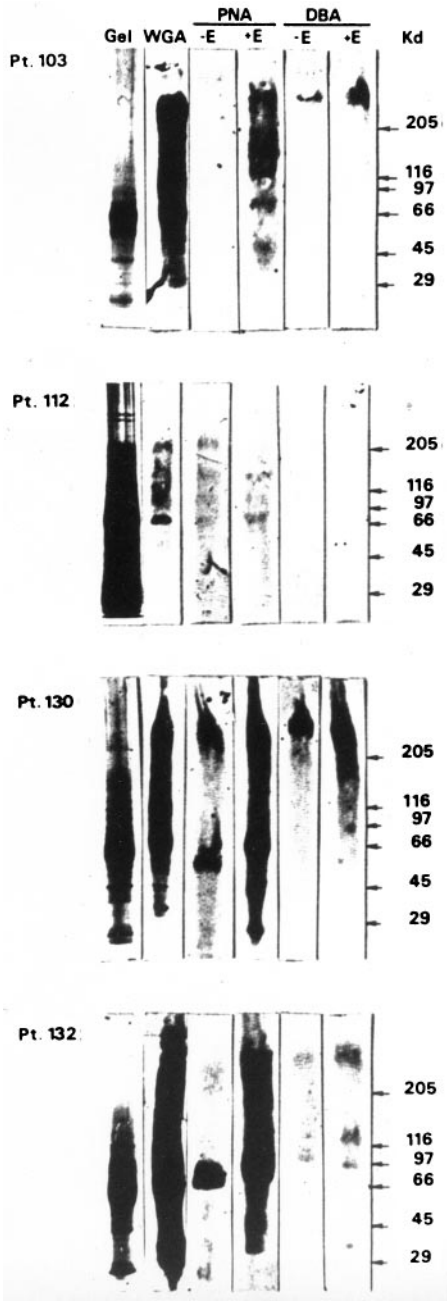

Figure 5. Lectin blots of crude human mucin from rectum, separated by SDS-PAGE, and transferred to nitrocellulose. Gel, Coomassie blue staining of gel; WGA, Lane stained with WGA; PNA, Lane stained with PNA; DBA, Lane stained with DBA; ± E, Without or with neuraminidase treatment before staining with lectin. kDa, Molecular mass markers in kilo daltons. Pt. 103, Normal rectum; Pt. 112, Rectum carcinoma, titration curves shown in Figure 1A and B. Pt. 130, rectum carcinoma, titration curve shown in Figure 1A. Pt. 132, rectum carcinoma.

Figure 5). A strong staining with both WGA and PNA on the blots, equalled a high titre tray signal (*eg* sample Rs 130), a moderate staining equalled a moderate signal (*eg* sample Rs 112).

Examination of rectal secretion from patients with benign and malignant diseases

The WGA-PNA.HRP assay gave low values with samples from normal individuals, individuals with benign diseases, and individuals cured for cancer (Figure 6). Only one individual, who was scored as cured from cancer by the clinicians, showed a high value. It could be suspected that this individual was actually not cured.

Individuals with limited colorectal cancer (Dukes A), showed mostly low values (three out of four), whereas most (eight out of ten) Dukes B and C individuals showed higher levels of binding (Figure 6), significantly different from those with benign disease ($p < 0.003$) and normals ($p < 4 \times 10^{-5}$). Interestingly, the group of patients with urological-, gynaecological- and various other cancers also showed increased levels. This was significant for gynaecological cancer compared with normals ($p < 2 \times 10^{-5}$) and benign disease ($p < 0.005$), and for the mixed cancer group compared with normals ($p < 6 \times 10^{-6}$) and benign diseases ($p < 3 \times 10^{-4}$). The highest values were recorded from patients with endometrial cancer, and a patient with chronic myeloid leucaemia.

The WGA-DBA.HRP assay showed another binding pattern, as normal, benign and cured cancer patients had the

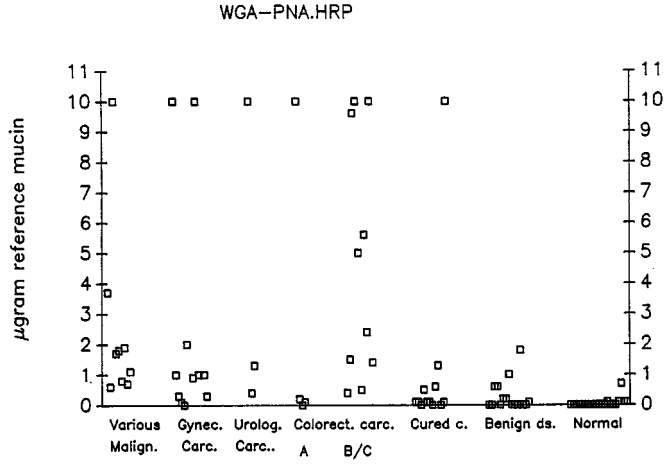

Figure 6. PNA binding to human rectal secretion captured by WGA in titre trays. Secretion from normals and benign diseases shows low binding, as does secretion from patients with cured cancer. Secretion from patients with colorectal cancer Dukes B/C, and patients with non-colonic cancers shows a high binding. The patient groups were: Normal: 18 individuals who underwent rectoscopy for abdominal complaint without any clinical or laboratory findings. Benign Disease: seven individuals with rheumatoid arthritis, five with colitis ulcerosa, four with mb Crohn. Cured cancer: four supposedly cured from colonic cancer, one supposedly cured from cancer of rectum, three supposedly cured from cancer of urinary bladder, three supposedly cured from uterine cancer, one supposedly cured from ovarian cancer. Colorectal cancer: Dukes A 10, Dukes B/C 11. Urological cancer: four with bladder cancer. Gynaecological cancer: eight cervical cancers, one endometrial cancer, three ovarian cancers. Various malignancies: one pleural mesothelioma, two malignant lymphoma, one carcinoid, four lung cancers.

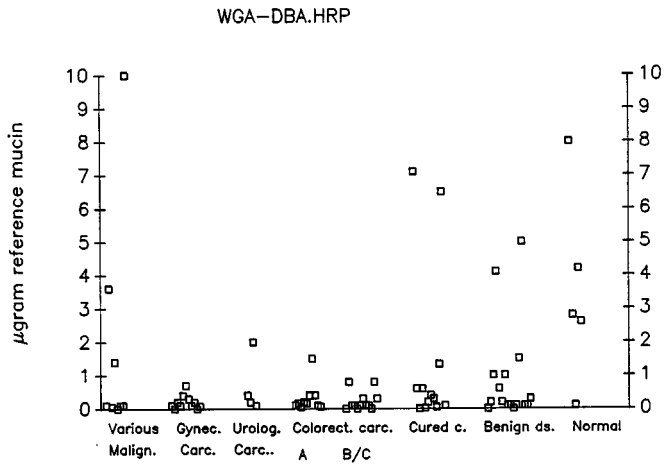

Figure 7. DBA binding to human rectal secretion captured by WGA in titre trays. Secretion from normals, patients with benign diseases, and patients with cured cancer show high binding levels, patients with cancer of colorectal or other origin show a low binding level.

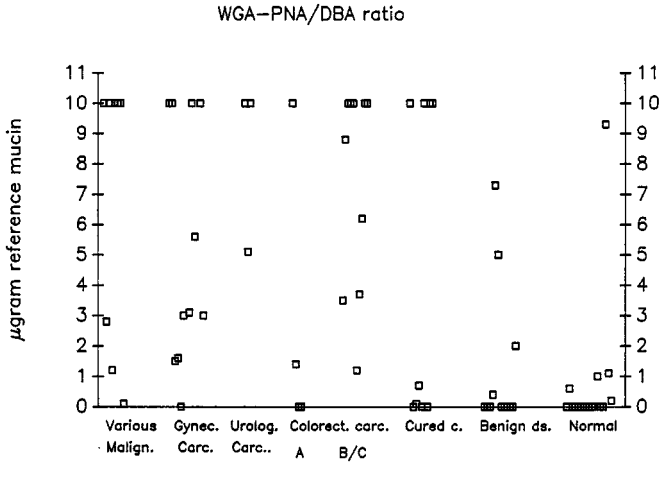

Figure 8. The calculated ratio between PNA and DBA binding to human rectal mucin captured by WGA in titre trays. The ratio gives a good separation between normals/benign diseases and cancer, irrespective of the cancers location in the body.

highest values, and cancer patients the lowest values (Figure 7). Due to this we calculated the WGA-PNA/DBA ratio (Figure 8), and found that this calculation only slightly improved the sensitivity for detecting cancer patients. The significance levels were similar to those obtained with the WGA-PNA.HRP assay. However, the predictive value of a negative test was better with the calculated PNA/DBA ratio, 84.6% compared with 72.3% for the WGA-PNA.HRP assay. Both assays had a positive predictive value of 87.2%. Analysis of the patients in Figure 8 showed a sensitivity of 72.3% and a specificity of 87.2% for the WGA-PNA assay, and a sensitivity of 87.2% and specificity of 84.6% for the WGA-PNA/DBA ratio. The PNA-WGA.HRP assay showed an ability to detect cancer patients quite similar to the WGA-PNA.HRP assay, however, as demonstrated above, this assay combination was inferior in terms of variation and recovery.

Discussion

Cells in a tissue will, at a certain time of development or under certain biological conditions, have prevailing glycosylation pathways [17, 18]. These pathways lead to biosynthesis of several different glycoproteins that may share mutual carbohydrate sequences in their glycan part. In the assay described in the present paper, different molecules are measured, provided that they have at least two carbohydrate sequences in common, one binding to the capture lectin, the other to the labelling lectin. Therefore, the assay described is well suited for studies of the glycan part of glycoproteins, and the changes occurring in these, ignoring that these may be present on different molecules. An advantage obtained by focusing on the glycans, is that the detection of even small quantitative changes in lectin binding in a tissue could become possible, due to the inclusion of not only one, but several different molecules as targets. In the present paper, human rectal secretion was applied, and by quantitating the amount of PNA binding structures on molecules immobilized by WGA, it was evident that rectal secretion from individuals with carcinomas showed significantly higher levels than secretion from normals and benign controls. Other combinations of lectins or anticarbohydrate antibodies might be useful in other applications of the method.

Materials like saliva, sweat, urine and rectal secretion are not subjected to a homoeostatic regulation as that found in plasma. This is a fact one has to consider when evaluating assays based on these systems. In the present assay the molecules detected in the material may vary from individual to individual. However, the examination of a number of normal, benign and malignant samples by use of the assay demonstrated its possible clinical usefulness.

Rectal secretion from normal individuals was only reactive in the assay after treatment with neuraminidase. This indicates a substitution of the PNA binding structures by sialic acid as found in O-linked structures SAα2-3Galβ1-3 [SAα2-6]GalNAcα1-O-R [19]. A similar structure is found in bovine submaxillary mucin [20], apart from the lack of α2-3 linked sialic acids in submaxillary mucins [19]. The DBA binding to the rectal secretion was higher in normal than in cancer individuals, and may occur to complex lactoseries extensions of the mucin core-structures [19].

The Western-blots showed binding of WGA to a wide range of glycoproteins present in human rectal secretion. The smear present in most lanes suggest the target molecules to be mucins. After desialylation PNA bound to an equally wide range of molecules. The blotting method does not allow to conclude that bands with similar molecular

weights are identical molecules. However, this conclusion can be reached by the present two-site assay, as only molecules which bind both WGA and PNA show positive reaction. A high molecular weight fraction of app. 250 kDa was the most reactive fraction with WGA, PNA, and DBA, indicating that this subfraction of glycoproteins in the rectal secretion is most susceptible to changes associated with the malignant process in the rectum.

Validation of the assay included determination of coefficients of variation. These determinations were carried out at the calibrated level around which the assay discriminates between rectal secretion from normal individuals and individuals with rectal carcinomas (WGA-PNA.HRP: 0.4; WGA-DBA.HRP: 0.65; PNA-WGA.HRP: 0.9). From a clinical point of view this is the level where maximal precision is desirable, if the assay objective is identification of cancer bearing patients. At this level, the intra-assay coefficient of variation was less than 7.5%, and the inter-assay variation coefficient was less than 10%, which is acceptable for clinical use. The analytical recovery was acceptable both in the assay where variable amounts were added to a standard amount, and in the dilutional experiments of high and low, and mixed high and low samples.

A problem solved in the present method was the selection of a standard that would be easily available, reactive in all assays, and having a glycan part similar to human rectal mucin. The bovine submaxillary asialomucin fulfilled these criteria and showed a good linearity in dilutional experiments. The oligosaccharide inhibition studies demonstrated highly similar binding specificity of the lectins to both the bovine submaxillary asialomucin standard and the crude human rectal secretion.

The recovery studies could not be performed with addition of patient mucin to bovine submaxillary asialomucin as mixtures of mucin from the two species behaved arbitrarily. This could be due to aggregation of mucin from the two species, but this or other explanations have to be elucidated in the future.

One-site glycan detecting assays involving purified glycoconjugates [21] or sera [22, 23] directly adsorbed onto plastic trays have been published. However, the reproducibility of these assays could be hampered by the fact that the direct adsorption of biological fluids to a plastic surface is a critical event that is difficult to standardize. This drawback is eliminated in the present two-site glycan detecting assay.

Another recent approach has been a competitive assay based on binding of lectin to enzyme conjugated monosaccharides in solution [24]. This approach seems particularly useful for the determination of lectin-saccharide reaction kinetics.

In conclusion, we believe the present assay to be well suited for simultaneous determination of two different glycans on a molecule. The combination of a lectin or antibody directed against a constant part, and one against a variable part of the glycans on a molecule showed good assay characteristics, when examining human rectal secretion. The assay ignores the specific nature of the involved molecules as it focuses only on the glycans. This could improve the sensitivity for detection of minor changes in glycosylation by detecting more molecules. Examination of a large number of clinical specimens showed the assay to be well suited for the detection of cancer, surprisingly not only cancer of the colon and rectum, but also cancer at other sites. We expected cancer of the colon and rectum to show altered glycosylation, as it is well known that there are glycosylation changes in the colonic mucosa far from the cancer. However, we did not expect diseases like endometrial cancer, or malignant lymphoma to lead to an altered glycosylation in rectal secretion. It is well known that cancers cause altered hepatic function with increased output of fibrinogen and chronic phase reactants, mediated by cytokines such as interleukin-1 and tumour necrosis factor [25]. Recent data indicate to us that, in patients with remote cancers, the glycosylation in rectal secretion is normalized if the patient is treated with steroids (data not shown). Thus, it seems to be a mechanism that can be downregulated by steroids, which is an effect also observed in some cytokine dependent mechanisms.

There have also been other reports on biological alterations in organs remote from the carcinomas, *eg* bucchal mucosa cells are altered when cancer is present [26], serum α1-3-fucosyltransferase activity was elevated in patients with gastric cancer and could be used for follow-up of the patients [27] and serum α1-3-fucosyltransferase activity was increased in 142 out of 169 patients with various cancers, independent of its location [28]. Finally, red blood cells lose their Lewis antigens when the patient has disseminated cancer [29]. Our findings are in concert with these reports; however, we still need a biological explanation for the altered activity of glycosyltransferases, and the altered protein glycosylation we observe in cancer patients.

References

1 Hakomori S (1989) *Adv Cancer Res* **52**: 257–331.
2 Feizi T (1985) *Nature* **314**: 53–7.
3 Hanisch FG, Uhlenbruck G, Dienst C, Stottrop M, Hippauf E (1985) *Eur J Biochemistry* **149**: 323–30.
4 Magnani JL, Steplwski Z, Koprowski H, Ginsburg V (1983) *Cancer Res* **43**: 5489–92.
5 Kannagi R, Fukushi Y, Tachikawa T, Noda A, Shin S, Shigeta K, Hiraiwa N, Fukuda Y, Inamoto T, Hakomori S, Imura H (1986) *Cancer Res* **46**: 2619–26.
6 Rosen SD (1993) Robert Feulgen Lecture 1993. *Histochemistry* **100**: 185–91.
7 Boland CR, Montgomery CK, Kim YS (1982) *Proc Natl Acad Sci USA* **79**: 2051–5.
8 Ørntoft TF, Mors NO, Eriksen G, Jacobsen NO, Poulsen HS (1985) *Cancer Res* **45**: 447–52.

9 Yonezawa S, Nakamura T, Tanaka S, Sato E (1982) *J Natl Cancer Inst* **69**: 777–85.
10 Sakamoto J, Furukawa K, Cordon-Cardo C, Yin BWT, Rettig WJ, Oettgen H, Old LJ, Lloyd KO (1986) *Cancer Res* **46**: 1553–61.
11 Ørntoft TF, Harving N, Langkilde NC (1990) *Int J Cancer* **45**: 666–72.
12 Campbell BJ, Finnie IA, Hounsell EF, Rhodes J (1995) *J Clin Invest* **95**: 571–6.
13 Denk H, Tappeiner G, Holzner JH (1974) *Eur J Cancer* **10**: 487–90.
14 Nagata Y, Burger MM (1974) *J Clin Invest* **249**: 3116–22.
15 Lotan R, Skutelsky E, Danon D, Sharon N (1975) *J Biol Chem* **250**: 8518–23.
16 Etzler ME, Kabat EA (1970) *Biochemistry* **9**: 869–77.
17 Schachter H (1986) *Biochem Cell Biol* **64**: 163–81.
18 Ørntoft TF (1990) *APMIS* **98 (suppl.17)**: 1–34.
19 Sadler JE (1988) In *Biology of Carbohydrates*, *vol.* 2. (Ginsburg V, Robbins PW eds,) pp. 200–88. New York: John Wiley and Sons.
20 Tsuji T, Osawa T (1986) *Carbohydrate Res* **151**: 391–402.
21 McCoy JP, Varani J, Goldstein IJ (1983) *Analytical Biochemistry* **130**: 437–44.
22 Ching CK, Rhodes JM (1989) *Br J Cancer* **59**: 949–54.
23 Shengle Z, Yiran L, Jinglue L, Yiran D, Di H (1990) *Cancer Biochem Biophys* **11**: 211–19.
24 Kim B, Cha GS, Meyerhoff ME (1994) *Anal Biochem* **218**: 14–19.
25 Andus T, Holstege A (1994) *Acta Gastroenterol Belg* **57**: 236–44.
26. Ogden GR, Cowpe JG, Green MW (1990) *Cancer* **65**: 477–80.
27 Yazawa S, Asao T, Nagamachi Y, Abbas SA, Matta KL (1989) *J Cancer Res Clin Oncol* **115**: 451–5.
28 Yazawa S, Madiyalakan R, Izazwa H, Asao T, Furukawa K, Matta KL (1988) *Cancer* **62**: 516–20.
29 Langkilde NC, Wolf H, Ørntoft TF (1991) *Br J Haematol* **79**: 493–9.

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