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An improved ELISA for the determination of sialyl Lewis^x structures on purified glycoconjugates

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The membrane carbohydrate antigen, sialyl Lewis x (sLe^x), is involved in cellular adhesive interactions in many diseases, such as cancer, inflammation and thrombosis. This antigen is also found on soluble macromolecules, such as serum glycoproteins, but the precise role of soluble sLe^x in modifying disease processes, or reflecting the pathological changes is still unclear. Although methods were previously reported for the measurement of soluble sLe^x, many of these were not well characterised, measurements were mainly made on mixtures of molecules, and the anti-sLe^x antibodies were used at concentrations that made the assay expensive. In this study an ELISA has been devised that detects sLe^x in purified soluble glycoconjugates using the anti-sLe^x antibody, CSLEX 1. Commercially-available haptoglobin (Hp) and synthetic complexes of Lewis antigens with polyacrylamide were used as model substances in developing the procedure. Key steps were washing the antibody/antigen complex with ten times diluted salt solution to prevent dissociation of the complex and the use of bovine serum albumin for blocking non-specific interactions. The assay was shown to be very specific, its precision was in the range 6–12%, and it could detect less than a pmol of sLe^x. It could also distinguish between different densities of sLe^x on the same amount of glycoconjugate. Determination of sLe^x in Hp isolated from small groups of healthy individuals, cancer patients, and rheumatoid arthritis sufferers suggested that the antigen expression is increased in disease. This method, which is an improvement on those previously described, will be useful for determining sLe^x in many different types of soluble glycoconjugate, and used in combination with synthetic carbohydrate polyacrylamide complexes, will help to standardize measurements of soluble sLe^x in the future.

Keywords: sialyl Lewis^x, haptoglobin, synthetic glycoconjugates, ELISA

Introduction

Sialyl Lewis^x (N-acetyl neuraminic acid α2-3Galactose β1-4(Fucose α1-3)N-acetylglucosamine) is a carbohydrate antigenic determinant that is found on the membranes of various cell types and on O- and N-linked oligosaccharide chains of some soluble glycoconjugate macromolecules (see [1–3] for reviews). This antigen is a ligand for the selectin family of adhesion molecules, and in this respect, it is important in leukocyte trafficking, inflammation, thrombosis and probably in the metastasis of some tumour cells. Increased sLe^x expression occurs in many tissues as part of the pathophysiological process and treatment with soluble sLe^x can inhibit inflammation-induced tissue damage.

The majority of studies of sLe^x in disease have investigated its expression on cells and tissue using immunocytochemistry [4–11]. Relatively little is known about the expression of sLe^x on individual macromolecules in body fluids. A few studies have reported on the expression of sLe^x in whole serum [12–16] and other body fluids [17, 18]. In many of these studies, the assay was poorly characterized, indirect and not easy to reproduce, and in other studies high amounts of the anti-sLe^x antibody were used and/or a primary antibody that required conjugation to a radioactive or enzyme label in order to achieve high degree of sensitivity in the assay.

Previously, we showed changes in the fucosylation and sialylation of α-1-proteinase inhibitor (API) and haptoglobin (Hp) in inflammation [19] and cancer [20]. As these carbohydrates are the important components of the sLe^x tetrasaccharide, it was necessary to determine if some of these changes were due to alterations in the sLe^x content. The aim of this study was to develop a multiwell

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assay to determine the expression of sLe^x on individual serum glycoproteins using the commercially-available monoclonal antibody, CSLEX1 [4]. The experimental conditions for the assay were established using the purified serum glycoprotein, Hp, and synthetic carbohydrate conjugates. To test the validity of the assay for clinical specimens, the expression of sLe^x was measured on Hp prepared from a small group of healthy individuals and patients with ovarian cancer or rheumatoid arthritis (RA).

Materials and methods

Blood samples were obtained by venepuncture from four women and four men (age range 33–64 years) with no known disease ('healthy'), six women with ovarian cancer and two women with breast cancer (age range 49–66 years), and five women with seropositive RA (age range 53–72 years). Sera were separated by low speed centrifugation (600 × g) and stored at −20 °C until required for analysis. The healthy individuals were attending a blood donor session and none were taking oral contraceptive or any other form of medication. Ovarian cancer was diagnosed by laparotomy (stages II–IV, FIGO classification) and histology (serous or mucinous adenocarcinoma), and all patients were receiving chemotherapy. At the time blood specimens were taken from the ovarian cancer patients, three patients were not responding to treatment, two were in partial remission and one specimen was from a patient who was in complete remission. Both breast cancer patients had very advanced disease with metastasis. All the RA patients had moderate disease activity as defined by the American Rheumatism Association, and all were receiving some form of medication. All specimens, except one healthy were from non-smokers, and in all cases the alcohol intake was very low.

Hp was isolated from sera by an affinity chromatography method [21]. Briefly, rabbit anti-human Hp antibody (Binding Site Ltd.) was coupled to CNBr-activated Sepharose beads (Pharmacia Ltd.) at a concentration of 3 mg antibody (Ab)/ml beads. A 250 μl aliquot of serum was mixed with 500 μl of Ab coupled beads, incubated for 1.5 h at room temperature, and unbound proteins were removed by washing nine times with 2 ml 25 mM Tris-HCl (pH 8.0) containing 140 mM NaCl, 1 mM $CaCl_2$, 0.05% (w/v) Nonindet P40 and 0.05% (v/v) phenyl methyl sulphonyl fluoride. After briefly washing the beads with deionised water to remove the salts, the bound Hp was eluted with 1 ml of 0.1 M trifluoroacetic acid. The concentration of purified Hp was determined by rocket electrophoresis [22] and by direct ELISA using a peroxidase conjugate of the above anti-Hp antibody. The purity of preparations was checked by SDS-PAGE in a Laemmli buffer system and visualized by silver staining [23]. The yield varied between 40 μg and 80 μg.

The final protocol used to measure sLe^x is shown below. The particular conditions chosen were established in pilot experiments. Multiwell plates from two manufacturers (Immunolon, Dynatech; Maxisorb, Nunc) were initially investigated, but the plate from Nunc gave a higher net absorbance in the assay, and was therefore used for all the subsequent studies. The amounts of first and second antibodies used were established by testing the former in the concentration range of 25–200 ng per well and the latter in the range of 0.39–50 ng per well. The results from other preliminary experiments are described in Table 1 in the Results section.

(a) To each well in triplicate was added 100 μl of 25 mM Tris-HCl/100 mM NaCl, (pH7.5) (TBS) containing 5–100 ng of pure Hp (Sigma Chemical Co. Ltd), 50 ng of Hp isolated from 'healthy' or 'diseased' serum, 0.39–50 ng (0.32–41.0 pmol) of Lewis antigens (sLe^a 20% mol, Le^x 20% mol, sLe^x 10% mol, sLe^x 20% mol, Syntesome GmbH, Munchen) coupled to poly(N-(2-hydroxyethyl)acrylamide) via a $-CH_2-CH_2-CH_2-$ spacer arm (molecular weight 30–40 kDa; carbohydrate composition of the conjugates confirmed by methanolysis

Table 1. Establishing optimum experimental conditions for the sLe^x assay

		CSLEX1 reactivity with glycoconjugate (absorbance 450 nm)				
Procedure	*Buffer Composition*	*Hp*	*sLe^x 20%*	*sLe^a 20%*	*Le^x 20%*	*CY*
1	TTBS	0.08 ± 0.02	0.04 ± 0.02	0.02 ± 0.02	0.02 ± 0.02	0.01 ± 0.01
2	TTBS-5	0.43 ± 0.03	1.44 ± 0.11	0.49 ± 0.07	0.47 ± 0.07	0.54 ± 0.14
3	TTBS-10	1.14 ± 0.09	1.15 ± 0.05	0.53 ± 0.04	0.50 ± 0.02	0.55 ± 0.07
4	TTBS-20	1.08 ± 0.02	1.15 ± 0.04	0.78 ± 0.03	0.87 ± 0.04	0.75 ± 0.03
5	TTBS-10B	0.82 ± 0.04	1.78 ± 0.04	0	0	0

The wells were coated with 50 ng of each of the above preparations as described in the text. In procedures 1–4, plates were blocked with TTBS, and all washing steps and antibody treatments were carried out in TTBS, or different dilutions of TTBS (1/5, 1/10, or 1/20) as shown. In procedure 5 the plate was blocked with TBS containing 0.5% BSA, washed with TTBS-10, and treated with antibody solutions containing TTBS-10 and 0.25% BSA. Each value in Tables 1 and 2 is the mean ± SD of 3–5 determinations. All values have had the background absorbance subtracted.

followed by gas chromatography and NMR), 50 ng of purified carboxypeptidase (CY) (Boehringer, Mannheim), or TBS alone. The plate was stood for 2 h at 37 °C. The concentrations of carbohydrate antigen in the sLe^a 20%, Le^x 20%, sLe^x 10%, sLe^x 20% were 0.81 μmol mg^{-1}, 0.91 μmol mg^{-1}, 0.63 μmol mg^{-1}, 0.81 μmol mg^{-1} respectively.

(b) After discarding the coating solution, the plate was washed four times with TBS, the residual liquid was removed by tapping the plate on tissue paper, 300 μl of 0.5% (w/v) bovine serum albumin (Sigma) in TBS were added, and the solution was stood for 2 h at 37 °C and 16 h at 4 °C.

(c) The plate was washed twice with TBS diluted ten times, containing 0.05% of Tween 20 (TTBS-10) and 100 ng of anti-sLe^x monoclonal antibody (Clone CSLEX1, Lot. 50023, Becton Dickinson) in TTBS-10 containing 0.25% BSA (TTBS-10B) were added. This mixture was incubated for 1 h at 37 °C.

(d) After washing five times with TTBS-10, 100 μl TTBS-10B containing goat anti-mouse IgM (10 ng per well conjugated with horseradish peroxidase, Sera Lab) were added and incubated for 1 h at 37 °C.

(e) The plate was again washed five times with TTBS-10, and 100 μl of 100 mM citrate buffer, (pH 6.0) containing 1 mM 3,3′,5,5′tetramethylbenzidine and 4.41 mM hydrogen peroxide were added to each well.

(f) The colour reaction was stopped by adding 100 μl of 12.5% (v/v) H_2SO_4 and the absorbance was determined at 450 nm in a plate reader (Titertek Multiskan, MCC/340).

Results

The mean background absorbance (without Hp or coating antigen, but with the blocking steps and antibody treatments) was 0.17 (SD = 0.02, 10 assays). All measurements were adjusted for background absorbance. The median intra-assay precision for 12.5 ng per well, 25 ng per well and 50 ng per well of commercial Hp from five assays was 5.9%, 9.1%, and 7.4% respectively (3–6 observations per assay). The median inter-assay precision for 12.5 ng per well and 50 ng per well of commercial Hp was 11.7% and 12.5% (12 assays). These values are similar to those previously quoted for a sLe^x ELISA [14, 15].

Table 1 shows results from experiments that were carried out to investigate the effect of buffer composition on the reactivity of the CSLEX1 antibody with commercial Hp and various negative (CY, Le^x 20% and sLe^a 20%) and positive controls (sLe^x 20%). When all the steps in the assay were carried out in undiluted TTBS very little antibody binding could be detected for any of the substances tested (procedure 1). When the plates were blocked with TTBS, but all the washing and Ab treatment steps were carried out in diluted TTBS, Ab binding was detected for all the coatings (procedures 2–4). The highest absorbance values were obtained for TTBS-10 and TTBS-20; however, the former was used in further experiments because it gave better discrimination between Hp and the negative and positive controls. In the fifth procedure, the plates were blocked with TBS containing 0.5% BSA, and 0.25% BSA in TTBS-10 was added to all antibody treatments; all the washing-steps being carried out with TTBS-10. Using these conditions Hp and sLe^x 20% gave high net absorbances and the absorbances for the negative controls were the same as the background values. Procedure 5 was used for all the remaining sLe^x measurements.

Figures 1 and 2 show the CSLEX1 binding after coating plates with different concentrations of commercial Hp or with different amounts of two of the synthetic sLe^x preparations (10% mol and 20% mol). All the substances tested showed a linear relationship between the amount material in the well and the absorbance value

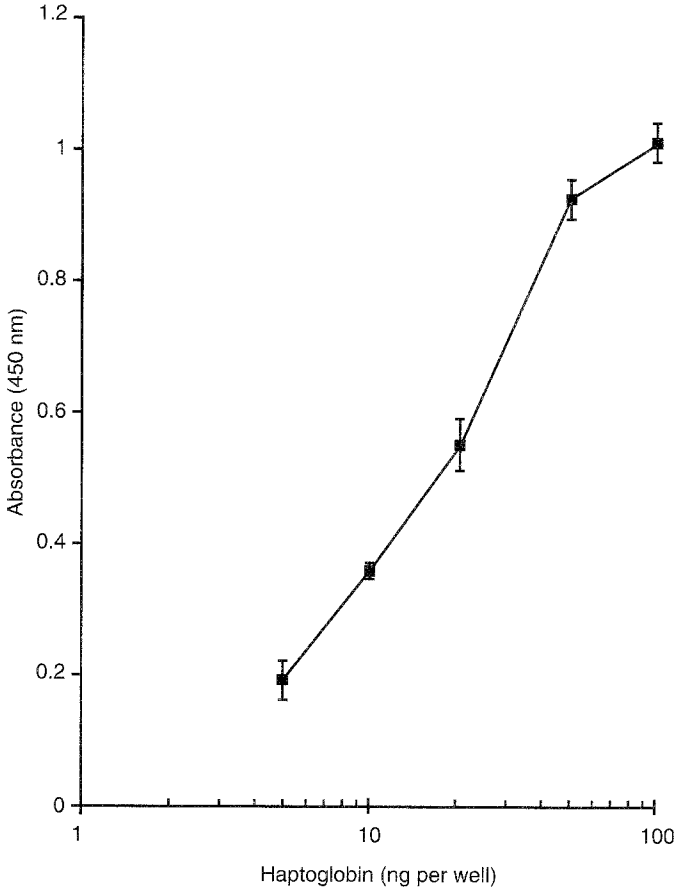

Figure 1. The reactivity of CSLEX1 antibody with different amounts of Hp coated on to a multiwell plate. Each value in Figs 1 and 2 is the mean ± SD of triplicate measurements.

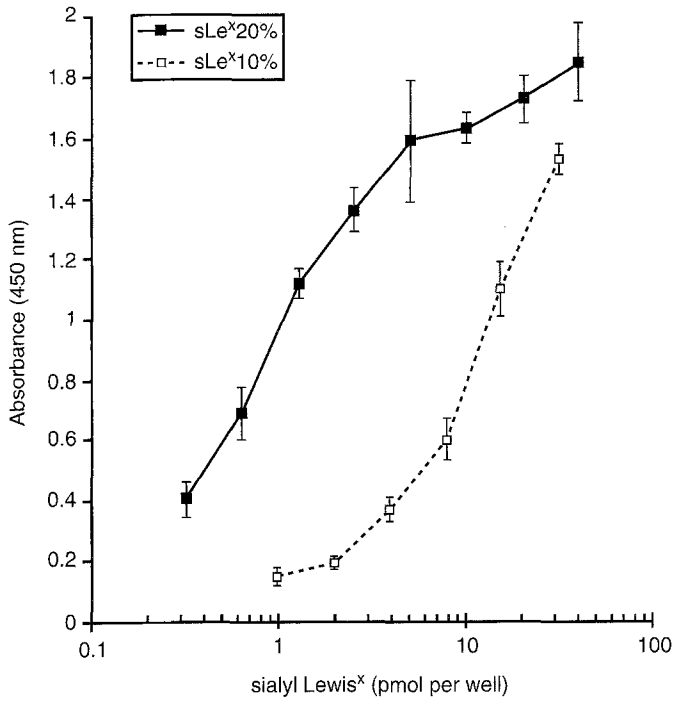

Figure 2. The reactivity of CSLEX1 antibody with different amounts of sLe^x coated onto a multiwell plate. Two different preparations of sLe^x were used (sLe^x 10% or sLe^x 20%), which differed in the density of antigen attached to polyacrylamide.

obtained. For Hp, sLe^x 10%, and sLe^x 20% the working ranges were 5–50 ng protein, 2–31.5 pmol sLe^x and 0.32–5.1 pmol sLe^x respectively. It was noted from Fig. 2 that the sLe^x in the '20%' preparation gave an absorbance value that was approximately five times higher than that given by '10%' preparation for a similar amount of added antigen. This difference, presumably, reflected the greater density of sLe^x on the former preparation.

Table 2 shows the binding of CSLEX1 to Hp isolated from the sera of eight healthy individuals, eight cancer patients and five RA sufferers. In the healthy group 6/8 specimens gave an absorbance value less than 0.5; whereas 7/8 cancer patients and 4/5 RA had an absorbance value greater than 0.5. The median absorbances for the healthy, cancer and RA groups were 0.40, 0.91, and 0.90 respectively. The very low value in cancer group was from a patient who was in complete remission. The two highest values in the healthy group were from a smoker and the oldest person in the study.

Discussion

This study has established an ELISA for the determination of sLe^x on soluble glycoconjugates, which is very sensitive, precise and specific. Previously reported ELISAs have been less satisfactory. Two of the best studies will be discussed in detail. In one [16], a plate was coated with 330 ng of the anti-sLe^x antibody (SH3) per well to capture glycoconjugates from 30 μl of serum to get a signal, that was detected by adding 8 μg radiolabelled antibody. These investigators expressed their results in terms of an undefined control serum and they did not present data for negative and positive glycoconjugate controls. We used 100 ng of antibody per well to detect sLe^x and have used precisely defined control glycoconjugates to characterise our assay. In another ELISA study [15], the sLe^x glycoconjugates in 50 μl serum were captured using 390 μg CSLEX1 antibody and the captured material was detected with the same antibody coupled to galactosidase. It was difficult to tell from this study amount of enzyme-labelled antibody used. No details were given on the non-specific interactions in this assay, and the study was standardized by using a supernatant prepared from stomach cancer cells.

One stimulus to start this study was the finding that the monoclonal antibody CSLEX1 could detect sLe^x on α-1-acid glycoprotein (AGP) by Western blotting [24]. Our previous attempts to blot Hp with this antibody were unsuccessful because the antibody/antigen complex dissociated during the washing procedure (unpublished observations); however, the AGP study showed that the complex remained stable if it was washed in a dilute salt solution [24]. Our current study agrees with this finding, and although this approach increases non-specific interactions, these can be blocked with BSA.

Another important observation from this study was the finding that polyacrylamide conjugated with sLe^x and other Lewis antigens could be coated on to a multiwell plate and used as controls. This allowed us to accurately confirm the specificity of the interaction of the antibody in the system that we developed. Furthermore, our results suggested that carbohydrate conjugates can be used to standardise ELISAs that measure carbohydrate antigens,

Table 2. sLe^x expression on serum Hp isolated from healthy individuals, cancer patients and rheumatoid arthritis sufferers

Specimen number	*Reactivity with CSLEX1 (absorbance 450 nm)*		
	Healthy	*Cancer*	*RA*
1	0.27 ± 0.06	[a]0.16 ± 0.02	0.46 ± 0.07
2	0.29 ± 0.06	[b]0.70 ± 0.03	0.85 ± 0.11
3	0.34 ± 0.02	[b]0.76 ± 0.15	0.90 ± 0.06
4	0.40 ± 0.03	[c]0.86 ± 0.15	0.98 ± 0.09
5	0.41 ± 0.03	[c]0.99 ± 0.13	1.34 ± 0.10
6	0.49 ± 0.02	[d]1.45 ± 0.06	
7	[e]0.84 ± 0.04	[d]1.52 ± 0.18	
8	[f]0.96 ± 0.03	[d]1.65 ± 0.06	

[a,b,d]ovarian cancer in complete remission, in partial remission, and progressive respectively; [c]breast cancer; [e]smoker; [f]64-years-old.

and in the future this approach will allow comparisons between different experiments, operators and laboratories. Previously, such comparisons were difficult to do because of variations in the methods of standardization used by different studies.

It did not appear from our results that the synthetic carbohydrate conjugates could be used to quantitatively measure the amount of sLe^x in a protein, because the sLe^x 20% gave a much higher signal than the sLe^x 10% for the same amount of antigen. Presumably, this was caused by the higher sLe^x density on the '20%' conjugate, which bound antibody with a greater affinity. This finding also indicates that previous studies which have reported differences in sLe^x content of glycoproteins in disease have to be interpreted with caution. An apparent elevation in sLe^x content may be due to an increase in antigen density rather than an absolute increase in antigen expression.

Although it is difficult to draw conclusions from the measurement of sLe^x on Hp isolated from the clinical specimens we used because of the low number of samples investigated, the overall increase in expression of this antigen in both cancer and inflammation agrees with previous findings with other serum glycoproteins [12–16, 24]. The high values we found for the oldest member of our healthy group and our only smoker suggest that factors other than disease may also be important in influencing sLe^x expression.

There is increasing interest in determining the glycosylation of molecules in relation to disease [19, 25] and function [26]. The currently-developed assay will help in both areas of study provided the glycoconjugate to be investigated is in a purified form. This purification can be done rapidly with clinical specimens using a batch affinity chromatography method that we previously developed, which has been successfully applied to the isolation of AGP [27], API [28], and Hp [21].

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