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Enzyme-linked immunosorbent assays for the measurement of blood group A and B glycosyltransferase activities

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ELISA assays have been developed for α(1-3)*N*-acetylgalactosaminyltransferase (blood group A transferase) and α(1-3)galactosyltransferase (blood group B transferase) activities. In these assays, microtitre plates coated with the bovine serum albumin conjugate of a synthetic Fucα1-2Galβ-R acceptor substrate are incubated with the appropriate nucleotide donor (UDP-GalNAc or UDP-Gal) and human serum as the enzyme source. The resulting trisaccharide products Fucα1-2(GalNAcα1-3)Galβ-R-BSA or Fucα1-2(Galα1-3)Galβ-R-BSA are detected and quantified with monoclonal antibodies selected not to cross-react with the substrate structure. With less than a microliter of human serum, product formation is proportional to enzyme concentration and to time of incubation of up to 90 min.

Keywords: ELISA, blood group A *N*-acetylgalactosaminyltransferase, blood group B galactosyltransferase, glycosyltransferase assays

The carbohydrate structures of human cell-surface glycoproteins and glycolipids are known to undergo systematic alterations during embryogenesis, cell-differentiation or malignant transformation (for reviews see [1–5]). Changes in the expression of the histo blood group ABO and Lewis antigens have been extensively studied in this regard, since these structures are readily detected using either monoclonal antibodies or lectins. Both *de novo* synthesis and disappearance of these antigens have been described but the regulation of their expression remains poorly understood. The most direct mechanism for controlling the expression of these antigens would be to regulate the expression of the glycosyltransferases required for their biosynthesis. Evidence has indeed been obtained that differential expression of specific glycosyltransferases can account, at least in some instances, for the observed differential cell surface antigen expression [6, 7].

The observation that altered expression of blood group glycosyltransferases can accompany cell transformation renders the development of rapid and specific assays for these enzyme activities an important objective. If changes in these activities can be detected in serum samples, such assays may be of diagnostic relevance. The human blood group A transferase (EC 2.4.1.40) catalyses the transfer of *N*-acetylgalactosamine from UDP-GalNAc to the H-precursor structure Fucα1-2Galβ-R (**1**) to give the A determinant Fucα1-2(GalNAcα1-3)Galβ-R (**2**) [8, 9]. The human blood group B transferase (EC 2.4.1.37) uses the same H acceptor, but UDP-Gal serves as the nucleotide donor for this enzyme which synthesizes the B determinant Fucα1-2(Galα1-3)Galβ-R (**3**) [8, 10]. Most published assays for these two enzyme activities quantify the rate of transfer of radiolabelled *N*-acetylgalactosamine or galactose from the sugar nucleotide donors to soluble acceptors [11]. When glycolipids are used as substrates, the products can be identified by mass spectrometry or by antibody staining of TLC plates [12]. We report here the development of nonradioactive ELISA assays for these activities which quantify the rate of conversion of immobilized substrates to A and B active products using monoclonal antibodies selected for this purpose. The structures of these products are thereby simultaneously verified, which is not the case in conventional radioactive assays.

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Materials and methods

Reagents

The synthetic BSA conjugates of the substrate disaccharide **1**, the blood group A and B trisaccharides **2** and **3** and Fucα1-2Galβ-O$(CH_2)_8$COOMe were obtained from Chembiomed Ltd, Edmonton, Canada. Human serum used as a source of A and B transferases was prepared by allowing freshly drawn blood to clot at room temperature for 2 h, refrigerating overnight at 4°C, and centrifuging to remove blood clots. NaN_3 was added to serum samples to give the final concentration of 0.01% (w/v); samples were stored frozen in 50 µl aliquots at −20°C. Affinity purified anti-A and anti-B monoclonal antibodies in 1% BSA in PBS were obtained from Chembiomed. These were screened for strong binding to the A or B trisaccharides, but not to the substrate disaccharide, following the procedure described in [13]. The alkaline phosphatase conjugate of goat anti-mouse IgM (µ-chain specific), alkaline phosphatase substrate tablets containing 5 mg *p*-nitrophenyl phosphate, UDP-Gal, UDP-GalNAc and Tween 20 were from Sigma, St. Louis, MO, USA. UDP-[6-^{3}H]galactose (15 Ci mmol^{-1}) was from American Radiolabeled Chemicals, St. Louis, MO, USA, and UDP-*N*-[1-^{3}H(N)]acetylgalactosamine (8.3 Ci mmol^{-1}) was from NEN-Dupont, Mississauga, Canada. EcoLite (+) liquid scintillation cocktail from ICN Costa Mesa, USA and Sep-Pak C-18 reverse phase cartridges from Waters (Mississauga, Ontario, Canada). Removable flat-bottomed wells of Immulon 2 were from Dynatech (supplied by Fisher, Edmonton, Canada). The following buffers were used: PBS, 7.8 mM Na_2HPO_4, 2.2 mM KH_2PO_4, 0.9% NaCl and 15 mM NaN_3, pH 7.4; and PBST, PBS with 0.05% Tween 20.

ELISA plate coating

Microtiter wells were coated as previously described [14, 15] by incubation with 100 µl synthetic BSA-glycoconjugate (20 µg ml^{-1}) in 50 mM sodium phosphate buffer, pH 7.5, containing 5 mM $MgCl_2$ and 15 mM NaN_3, for 16 h at ambient temperature. The solution was then aspirated and replaced with 5% BSA in PBS (200 µl). After 4 h, this solution was removed and wells were washed three times with PBS (200 µl) and once with H_2O (200 µl), air-dried for 1 h, and stored at 4°C. Plates were washed again with H_2O (200 µl) immediately before use.

Serum incubation

Transferase assays were performed by adding diluted serum (1/100 with assay buffer) and UDP-GalNAc or UDP-Gal, for the A and B transferase, respectively, in 100 µl of 50 mM sodium cacodylate buffer (pH 6.8) containing 14 mM $MnCl_2$ to microtiter wells coated with the BSA conjugate of **1**. This pH was determined to be optimal for B transferase activity and allowed for a side-by-side comparison of the A and B transferase activities. All assays were carried out in triplicate with the buffer composition, volume of serum, and nucleotide donor noted in the figure legends. The microtiter plate was incubated at 37°C for 60 min. The reaction mixture was removed by aspiration; wells were washed (2 × 200 µl H_2O and 2 × 200 µl PBST). Anti-product antibody was diluted 1/200 for anti-A and 1/1000 for anti-B in 1% BSA/PBST and 100 µl was added to the microtiter wells. After 2 h incubation at ambient temperature, the antibody solution was aspirated, wells were washed with 5 × 200 µl of PBST, and then incubated with alkaline phosphatase-conjugated goat anti-mouse IgM (100 µl of 1/1000 dilution in 1% BSA/PBST) for 2 h at ambient temperature. This solution was aspirated and wells were washed (3 × 200 µl PBST and 1 × 300 µl H_2O) before adding *p*-nitrophenyl phosphate solution, 1 mg ml^{-1} in 1 M diethanolamine-HCl buffer, pH 9.8, containing 1% BSA and 0.5 mM $MgCl_2$. The increase in absorbance at 405 nm with background correction at 650 nm to correct for any inhomogeneity between wells was monitored over time using a Molecular Devices Thermomax microplate reader. Data acquisition was controlled by a Macintosh SOFTmax program and the absorbance readings reported were taken after 60 min of colour development.

Sep-Pak radiochemical assays

These assays are based on modifications of a previously described method [16] which makes use of hydrophobic acceptors and products to facilitate the removal of unreacted radiolabelled donor from reaction products. Incubations for A and B assays were carried out in 1.5 ml plastic microfuge tubes at 37°C for 60 min and 90 min, respectively. The blood group B transferase assay was carried out in a total of 66 µl with 50 mM sodium cacodylate buffer, pH 6.8, containing 21 mM $MnCl_2$, 30 µM UDP-Gal, 0.2 µCi UDP-[6-^{3}H]Gal, 15–46 µM Fucα1-2Galβ-O$(CH_2)_8$COOMe and 25 µl of serum. At the end of the 90 min incubation period, samples were diluted with water (1 ml) and applied to pre-conditioned Sep-Pak cartridges which were washed with about 150 ml of water until background counts were obtained in the aqueous wash. Radiolabelled products were eluted with methanol (2 × 5 ml) and quantified as dis-integrations min^{-1} in 10 ml of EcoLite (+) scintillation cocktail in a Beckman LS 1801 counter. Substrate inhibition was observed at an acceptor concentration greater than 50 µM; data below this concentration were used to estimate V_{max} and K_M using the Wilkinson method [17]. The assay for the blood group A transferase was carried out with the same acceptor; in this case the assay volume was 100 µl, in 50 mM sodium cacodylate buffer, pH 6.8, containing 14 mM $MnCl_2$, 25 µM UDP-GalNAc, 0.3 µCi UDP-[^{3}H]GalNAc, 0.5 to 3.5 µM Fucα1-2Galβ-O$(CH_2)_8$COOMe, and 30 µl of serum. At concentrations greater than 3.5 µM, substrate inhibition was observed.

Results and discussion

The acceptor substrate **1** as well as products **2** and **3** were covalently attached to BSA through a 9-carbon linking arm [18] with incorporations of 16–23 oligosaccharides per BSA molecule. Standard mixtures with defined ratios of product and substrate conjugates were used to coat ELISA wells. These mixtures serve as reference standards to mimic plates that would result from the action of the A or B blood group transferase on the immobilized acceptor (**1**). Figure 1a,b shows the detection of various ratios of the product A and B determinants in admixture with **1** employing two different monoclonal antibodies. For the A determinant, Fucα1-2(GalNAcα1-3)-Galβ (**2**), the ELISA response is linear up to the equivalent of 10% product (Fig. 1a). For the B determinant, Fucα1-2(Galα1-3)-Galβ (**3**), the response is linear up to 0.5% product (Fig. 1b). These standard curves define the operational range of the assay, and can be used to compensate for day to day variations for comparison of absolute enzyme activity. While the slope of these standard curves vary when different lots of plates are coated or new reagents prepared, the linear range is invariant.

Addition of diluted blood group A serum and UDP-GalNAc to microtiter plates coated with the Fucα1-2Galβ-BSA conjugate resulted in the formation of immobilized

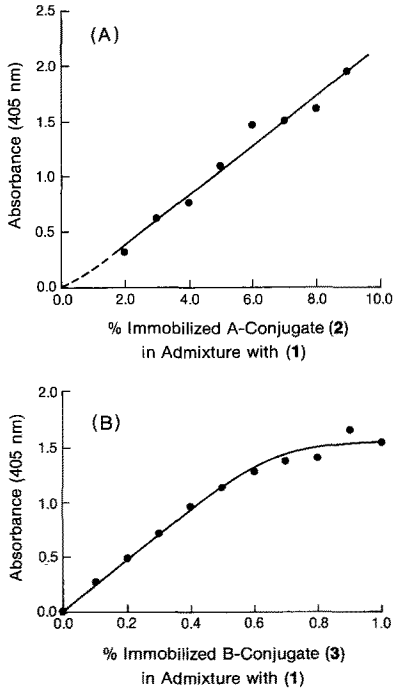

Figure 1. Standard curves for ELISA response of wells coated with increasing ratios of product to substrate. Antibody incubations were carried out as described in the text. (a) Standard curve for A transferase assay with increasing ratio of the product Fucα1-2(GalNAcα1-3)Galβ-R-BSA (**2**) to substrate Fucα1-2Galβ-R-BSA (**1**). (b) Standard curve for the B transferase assay with increasing ratio of product Fucα1-2(Galα1-3)Galβ-R-BSA (**3**) to substrate **1**.

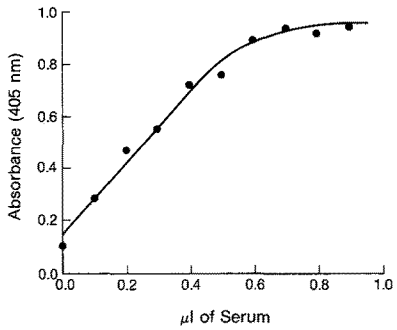

Figure 2. Effect of increasing enzyme concentration on blood group A glycosyltransferase product formation. Enzyme incubations were carried out for 1 h at 37°C with 25 μM UDP-GalNAc in 50 mM sodium cacodylate buffer with 14 mM $MnCl_2$, pH 6.8. Serum from type A individual was used as the source of A transferase.

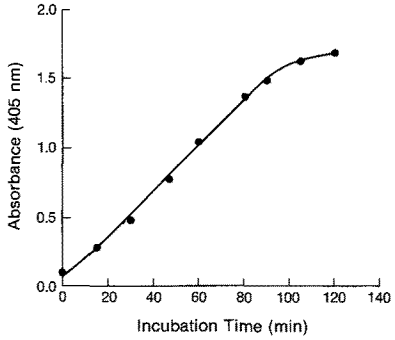

Figure 3. Effect of enzyme incubation time on ELISA response. Type A serum (0.9 μl) was incubated with 25 μM UDP-GalNAc in 100 μl 50 mM sodium cacodylate buffer, pH 6.8, containing 14 mM $MnCl_2$ for the times indicated.

Fucα1-2(GalNAcα1-3)-Galβ (**2**) as detected by the anti-A monoclonal antibody. As seen in Fig. 2, the amount of product formed is proportional to the amount of serum added for a 1 h incubation at 37°C. In Fig. 3 the amount of product formed is shown to be proportional to the time of incubation for a fixed concentration (0.9 μl) of serum. No product formation was detected in incubations that lacked the nucleotide donor UDP-GalNAc, or when type O or B serum was used. The sensitivity of the assay is comparable to other literature methods which employ radiolabelled UDP-GalNAc and quantify label incorporation in product. A standard solution assay for A activity with Fucα1-2Galβ-$O(CH_2)_8COOMe$ (K_M = 3.5 μM) as an acceptor required 30 μl of serum and 60 min of incubation to quantify activity, demonstrating that with this substrate the ELISA is more sensitive than a radiochemical method.

Figure 4 shows the formation of Fucα1-2(Galα1-3)-Galβ (**3**) when blood group B serum and UDP-Gal are incubated in microtiter plates coated with Fucα1-2Galβ-BSA conjugate. The amount of product formed is proportional to

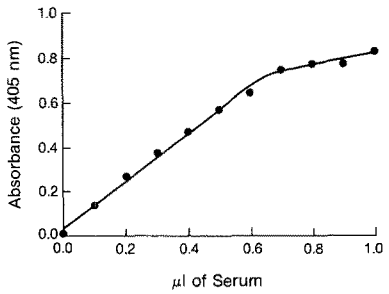

Figure 4. Effect of increasing enzyme concentration on blood group B glycosyltransferase product formation. Incubations were carried out for 1 h at 37°C with 20 μM UDP-Gal in 100 μl 50 mM sodium cacodylate buffer, pH 6.8, containing 14 mM $MnCl_2$. Serum from a type B individual was used as the source of B glycosyltransferase.

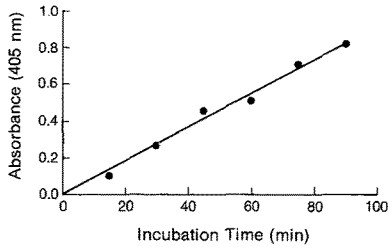

Figure 5. Effect of enzyme incubation time on ELISA response for the blood group B glycosyltransferase reaction. Incubations were carried out for the times indicated using 0.5 μl type B serum as the enzyme source and 20 μM UDP-Gal.

serum concentration up to 0.6 μl, for 1 h incubations. Figure 5 shows the effect of incubation time at a fixed enzyme level, where a linear response is observed up to 90 min. The ELISA method is also more sensitive than the radiochemical method for the blood group B transferase. This enzyme has a K_M of 88 μM for Fucα1-2Galβ-O$(CH_2)_8$COOMe, and 25 μl serum were required to detect activity in a 90 min incubation in the radiochemical method.

Figure 6 shows the results of screening a panel of blood typed serum samples. It can be seen that type O serum gives a low response, defined as background, for incubations with UDP-Gal and UDP-GalNAc developed with anti-A or anti-B antibodies (samples from donors WS, LK, MA, and EH). Samples of type A sera (MG, SC, LN, OY, MH and HG) have absorbances of 0.4 to 1.4 at 405 nm when incubated with UDP-GalNAc and developed with anti-A antibodies. These samples exhibit a low response in incubations with UDP-Gal followed by product detection with anti-B antibodies; however, there appears to be some crossreactivity giving values above background. This apparent crossover in reaction specificity has been studied and attributed to a slow reaction of the A enzyme with UDP-Gal to give Fucα1-2(Galα1-3)-Galβ- structures, the

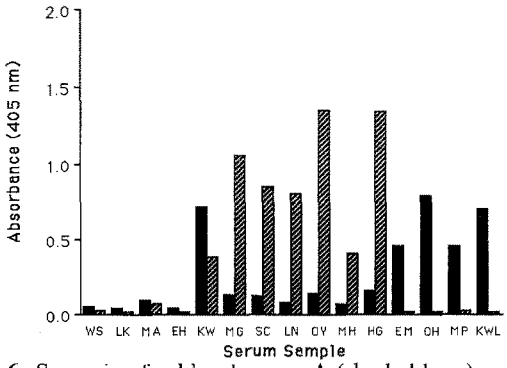

Figure 6. Screening for blood group A (shaded bars) and B (solid bars) transferase activities in serum samples from different donors. For A transferase activity a 1 μl serum sample was used and for B transferase activity a 0.7 μl serum sample was screened. Subjects WS, LK, MA, EH are type O; KW is an AB source; MG , and SC are A, LN, OY, MH, and HG are type A_1; EM, OH, MP and KWL are type B.

blood group B enzyme product [19]. A panel of B serum samples (EM, OH, MP and KWL) gave absorbances between 0.5 to 0.8 for incubations with UDP-Gal and product detection with anti-B antibodies. Although cross-overs in donor specificity have also been reported for blood group B enzymes [19] these are not apparent in our incubations with UDP-GalNAc developed with anti-A antibodies. As expected an AB subject (KW) exhibited both blood group A and B activity.

In summary, we have shown that a synthetic immobilized disaccharide conjugate can serve as an efficient acceptor for the blood group A and B specified glycosyltransferases. Product formation is linear with time for 1 h incubations using under 1 μl of serum as the enzyme source. The assays are at least as sensitive as conventional radiochemical assays, and the structures of the products are in addition characterized by the specificity of the monoclonal antibodies used. Immobilized acceptor BSA-conjugates were used in this study but this ELISA approach, originally developed by Stults, Wilbur and Macher [20], can equally well be employed using immobilized glycolipids. Several additional examples of the use of ELISA assays for the quantification of glycosyltransferase activities have since appeared [21–24], suggesting that this approach may be general.

Since ELISA assays are readily automated, the ability to screen large number of samples conveniently for A and B glycosyltransferase activities is at hand. The possibility that changes in these enzyme activities may correlate with the expression of aberrant cell surface ABO antigens can be evaluated for diagnostic significance. Preliminary data have suggested recently that quantification of A and B transferase activity may be useful in A_1 and A_2 subgroup typing [25]. However, any attempts to distinguish between these two enzyme activities must take careful consideration of potential differences in their pH optima [26, 27] and substrate specificity and competition [28].

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