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AN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE MEASUREMENT OF LEWIS BLOOD-GROUP α-(1→4)-FUCOSYLTRANSFERASE ACTIVITY

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ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) has been developed for the Lewis blood-group associated α-(1→4)-fucosyltransferase activity. Microtiter plates coated with the bovine serum albumin conjugate of a synthetic β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc disaccharide are incubated with a fucosyltransferase preparation in the presence of guanosine 5′-diphosphofucose. The resulting immobilized Lewis-a active trisaccharide β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-β-D-Glc*p*NAc is then detected and quantitated using a monoclonal anti-Lewis-a antibody. Product formation detected in this manner is linear with time, proportional to enzyme activity, and reproducibly quantitated in the 50–400 fmol range.

INTRODUCTION

The oligosaccharide chains of glycoproteins and glycolipids are biosynthesized through the concerted action of a large number of glycosyltransferases which catalyze the transfer of glycosyl residues from sugar-nucleotides to growing carbohydrate chains[1,2]. Changes in the completed oligosaccharide structures have long been known to accompany the progression of several diseases[3–5], especially cancers, but only recently have these changes been shown to correlate frequently with alterations in enzyme expression[6–11]. Glycosyltransferase activities have therefore been proposed as potential diagnostic markers of disease, but experimental corroboration of this proposal has remained largely elusive[12–16].

A major impediment to the evaluation of glycosyltransferase activities as

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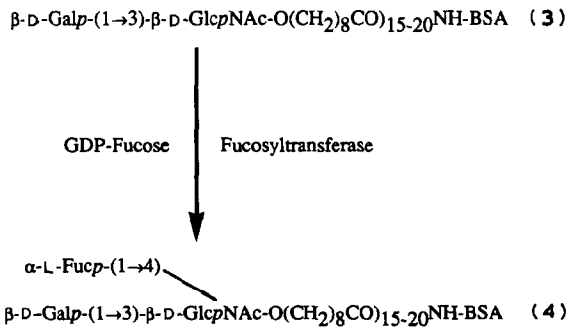
clinically relevant diagnostic markers has been the cumbersome nature of the enzyme assays. Almost all glycosyltransferase assays measure the rate of transfer of radiolabelled sugars from the sugar-nucleotide donor to an appropriate oligosaccharide acceptor which must usually be isolated from biological sources[1]. Time-consuming procedures, including h.p.l.c., gel-permeation chromatography, affinity chromatography, and/or high-voltage p.e., are generally required for the separation of unreacted sugar-nucleotide (and its degradation products) from the enzyme product before quantitation by liquid scintillation counting[1,6,17–21]. To further complicate these procedures, the natural oligosaccharide acceptors are frequently substrates for several enzymes, necessitating the separation and structural verification of the labelled enzymic products in each assay. Synthetic oligosaccharide analogs show a great deal of promise for alleviating these latter difficulties, since such structures can frequently be made "mono-specific" by removal or masking of interfering cross-reactive hydroxyl-groups[22–24]. When such synthetic acceptors are prepared as hydrophobic glycosides, the assays are further simplified because the labelled products can be rapidly isolated on reverse-phase (C-18) sample-preparation cartridges[25]. Synthetic acceptors for glycosyltransferases have an added advantage, since they can be made available in large quantity and the presence of unknown oligosaccharide contaminants is effectively eliminated.

Stults, Wilbur, and Macher[26] recently reported that isolated glycosphingolipids are readily immobilized by adsorption on plastic ELISA microtiter plates where they can serve as substrates for β-D-galactosyltransferase. The products of the enzyme-catalyzed reaction can then be detected by a "sequence-specific" carbohydrate antibody and the amount of product can be amplified linearly by conventional ELISA methodology and quantitated. Their report is the first demonstration that the ELISA technique can potentially serve as a routine glycosyltransferase assay with the potential for extensive automation and therefore for screening large numbers of samples. We now report that the advantages inherent in using synthetic substrate-analogs can be directly incorporated into an ELISA. The ELISA presented here is for the Lewis-fucosyltransferase, an enzyme that is readily obtained from human milk[27]. The enzyme catalyzes the transfer of fucose from GDP-fucose to Type 1 chains, β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-, producing the Lewis-a (Le^a) blood-group determinant β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-β-D-Glc*p*NAc. The synthetic Type 1 disaccharide β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-O$(CH_2)_8COOCH_3$ (**1**) has been shown to be a soluble substrate for this fucosyltransferase, producing the Le^a trisaccharide β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-β-D-Glc*p*NAc-O$(CH_2)_8COOCH_3$ (**2**)[22,28]. We now demonstrate that **3**, the synthetic glycoconjugate prepared by attachment of **1** to bovine serum albumin (BSA)[29], can be used to coat microtiter plates where it can also serve as an effective acceptor for the enzyme. Product **4** is then detected and quantitated by a monoclonal anti-Le^a antibody (CF4-C4) whose combining-site has been extensively probed using synthetic oligosaccharide analogs[30,31]. This antibody has an absolute requirement for HO-4 of the fucosyl residue in the Le^a-trisaccharide and, therefore, does not cross-react with unfucosylated **3**.



RESULTS AND DISCUSSION

Standard mixtures containing various known ratios of **3** and **4** were prepared and used to coat ELISA plates. The resulting wells provide a set of synthetic reference standards expected to simulate wells initially coated with **3** and which have undergone a known extent of enzymic fucosylation. Detection of the Le^a antigen on these plates by antibody CF4-C4 is shown in Fig. 1. The ELISA response is seen to be linear up to at least the equivalent of 5% fucosylation. This finding establishes a linear range intrinsic to the ELISA, and subsequent enzymic conversions should ideally fall within this range. In order to verify that the response of such synthetic mixtures of homogeneous antigens was indeed the same as that of the equivalent antigen produced by enzymic fucosylation, BSA was coupled with a 9:1 mixture of **1** and **2**. The resulting glycoconjugate was analyzed for galactose and fucose which were found to be present in the ratio 11.6:1, within experimental error of the expected ratio. As seen in Fig. 1, the response of wells coated with this mixed synthetic antigen very closely approximated that of the mixture, in the same ratio, of the homogeneous antigens **3** and **4**.

The advantage of using a set of synthetic reference standards representing known extents of fucosylation lies in the increased reproducibility of results from day to day, especially when new solutions of primary or secondary antibody are used. Thus, although variation can be expected in the absolute absorbances reported from different laboratories at different times, the use of a standard curve such as that in Fig. 1 should allow comparison of absolute enzymic activity.

Addition of GDP-fucose and fucosyltransferase to ELISA plates coated with the β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc glycoconjugate **3** resulted in the production of the Le^a antigen β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-β-D-Glc*p*NAc **4**, as detected by monoclonal antibody CF4-C4. The dependence of the rate of product formation on enzyme concentration is shown in Fig. 2 where it is seen to be linear up to ~0.6 absorbance unit (AU). The dependence of antibody-detected product formation on the time of incubation with fucosyltransferase is presented in Fig. 3 where it is again found to be linear up to ~0.6 AU, after which it levels off. It may be noted

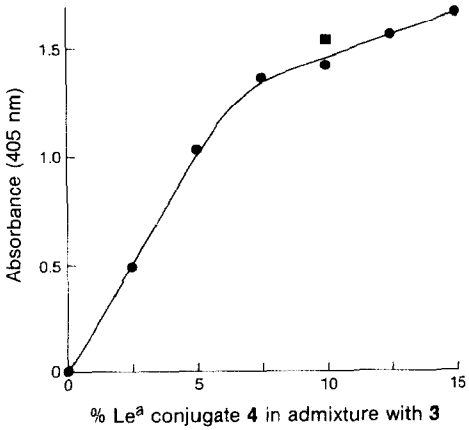


Fig. 1. ●, ELISA response of wells coated with various proportions of **3** and **4**. Absorbance was measured after an 80-min incubation with *p*-nitrophenyl phosphate. ■, Response of wells coated with a synthetic BSA-glycoconjugate prepared by using a 9:1 ratio of **1**:**2**.

that the response of the ELISA at very low levels of product formation (<30 fmol) is not necessarily linear, and caution should be exercised in attempting to work in this region.

The K_m for guanosine 5′-diphosphofucose (GDP-fucose) in the ELISA was 3.2 μM (data not shown). The fucosyltransferase reactions described above were performed in the presence of 6.1 μM GDP-fucose in order to conserve this reagent and since use of higher concentrations of nucleotide donor did not increase importantly the rate of product formation in the wells. Thus, 1 mg of GDP-fucose is sufficient for 2500 assays.

An estimate of the amount of fucosylated product formed and detected by antibody CF4-C4 was obtained by performing the incubation in the presence of ^{14}C-labelled GDP-fucose. The absorbances of individual wells were measured on

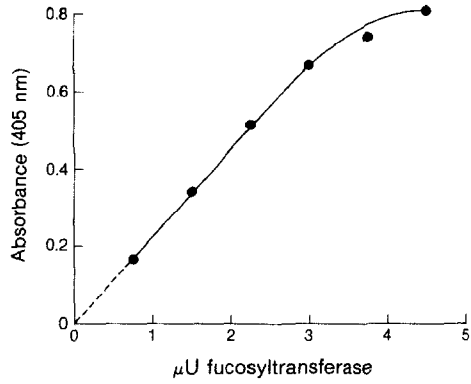


Fig. 2. Dependence of the ELISA response on fucosyltransferase activity. Plates coated with **3** were incubated with various amounts of fucosyltransferase in the presence of GDP-fucose for 60 min at 37°. Absorbance was measured after an 80-min incubation with *p*-nitrophenyl phosphate.

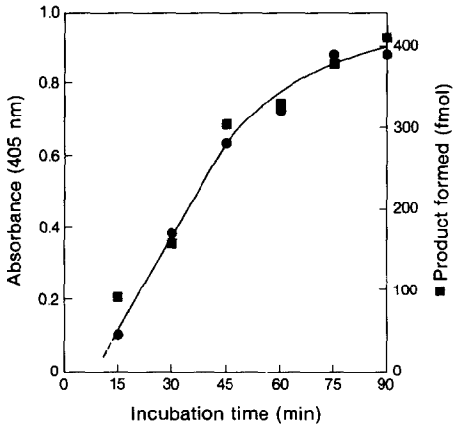


Fig. 3. Dependence of the ELISA response on incubation time with fucosyltransferase. ●, Wells coated with **3** were incubated with 3 μU of fucosyltransferase and GDP-[^{14}C]fucose for the indicated times at 37°. Absorbance was measured after an 80-min incubation with *p*-nitrophenyl phosphate. ■, After the absorbance measurement, the amount of ^{14}C-fucose in individual wells was determined by liquid scintillation counting.

the ELISA-reader and product formation in each well was then quantitated by liquid scintillation counting. The results presented in Fig. 3 show a reading of 1.0 AU produced in an 80-min ELISA development, to correspond to the formation of 440 fmol of product.

The sensitvity of this ELISA assay compares favorably to that of radiochemical methods. In the fucosyltransferase reaction, for radiochemical assays containing only radiolabelled GDP-fucose, the minimal amount of detectable fucose transferred would be of the order of 100 d.p.m. At a specific activity of 239 mCi/mmol, this corresponds to 190 fmol of fucose, in the middle of the linear range of the ELISA.

The ELISA described above is not restricted to the use of highly purified fucosyltransferase activity, since we have recently reported the detection of Lewis fucosyltransferase activity in mung-bean seedling microsomal extracts, using this assay[32]. The development of similar ELISAs, using synthetic acceptors and antibodies screened against synthetic glycoconjugates, is currently underway in our laboratories.

EXPERIMENTAL

Materials. — Human milk fucosyltransferase was purified and assayed by a modification[28] of reported[27,33] procedures, to a specific activity of 0.106 U/mg. Antibody CF4-C4, an IgG, was a gift from Dr. W. W. Young, Jr. and was affinity-purified as previously described[31]. Goat anti-mouse IgG (Fc specific) conjugated to alkaline phosphatase and tablets containing 5 mg of *p*-nitrophenyl phosphate substrate were obtained from Sigma. GDP-[U-^{14}C]fucose (239 mCi/mmol) was from

New England Nuclear. ACS liquid scintillation cocktail was from Amersham. Removable flat-bottomed microtiter wells of Immulon 2 were from Dynatech. BSA-conjugates **3** and **4** were available from previous work[29,32] and had incorporations varying between 15 and 20 mol of oligosaccharide/BSA molecule. The following buffers were used: PBS, 7.8mM Na_2HPO_4, 2.2mM KH_2PO_4, 0.9% of NaCl, and 15mM NaN_3 at pH 7.4; PBST, PBS containing 0.05% of Tween 20.

Methods. — Microtiter plates were coated by incubation with 100 μL of synthetic BSA-glycoconjugate (20 μg/mL) in 50mM sodium phosphate buffer (pH 7.5), containing 5mM $MgCl_2$ and 15mM NaN_3, for 16 h at ambient temperature. The solution was then removed by aspiration and replaced with 5% BSA in PBS (200 μL). After 4 h, this solution was removed, and the wells were washed four times with PBS (200 μL) and twice with H_2O (200 μL), air dried for 1 h, and stored at 4°. Plates were washed again with H_2O (200 μL) immediately before use. These plates coated with **3** or **4** are now commercially available [Chembiomed Ltd., product number 67-811P (**3**) and 62-301P (**4**)].

For fucosylation reactions, the enzyme and GDP-fucose (6.1μM) in 110 μL of 50mM sodium cacodylate buffer (pH 6.5) containing 12mM $MnCl_2$ were added to the coated wells. After selected incubation times at 37°, the solution was removed by aspiration, and the wells were washed with H_2O (200 μL) and PBST (200 μL). Antibody CF4-C4 (0.3 μg/mL in 1% BSA in PBST, 200 μL) was added to the wells. After 2 h, the solution was removed by aspiration, the wells were washed with PBST (5 × 200 μL), and alkaline phosphatase conjugated goat anti-mouse antibody (200 μL, 1/3000 dilution in 1% BSA in PBST) was added. After 2 h, the wells were washed with PBST (3 × 200 μL), H_2O (200 μL), and H_2O (300 μL). *p*-Nitrophenyl phosphate [1.0 mg/mL in M diethanolamine–HCl buffer (pH 9.8) containing 1% of BSA and 500μM $MgCl_2$ (100 μL)] was then added and the increase in absorbance at 405 nm was monitored with a Bio-Tek EL-309 EIA plate reader. All assays were performed at least in triplicate and the average readings which varied <15% are reported. In ELISA experiments involving the formation of radiolabelled product, GDP-^{14}C-fucose (341,000 d.p.m. and total GDP-fucose 11 μM) was included in each well. After the absorbances of the wells had been measured, the contents were transferred to scintillation vials and counted in 10 mL of ACS cocktail. Individual wells contained between 50 and 145 d.p.m. with a background of 25 d.p.m. (Fig. 2).

The BSA-conjugates were prepared according to Pinto and Bundle[34]. For mixed antigens, the acyl azides[34], β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-O$(CH_2)_8CON_3$ and β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-β-D-Glc*p*NAc-O$(CH_2)_8CON_3$ were prepared as 23mM solutions in $HCONMe_2$ at −15° and were then mixed in a ratio of 9:1 in a total of 100 μL. To this mixture was added BSA (5.2 mg) in 5 mL of 0.35M $KHCO_3$ and 0.08M $Na_2B_4O_7$ (pH 9.0). The solution was kept at 4° for 18 h, then exhaustively dialyzed (Amicon PM-10 membrane) against distilled water, and lyophilized. The carbohydrate content of the residual white powder was determined, using the phenol–sulfuric acid assay[35], and yielded an incorporation of 20

oligosaccharides per BSA molecule. Protein was quantitated by the Bradford assay method[36], using BSA as a standard. Independent analysis for both D-galactose and L-fucose was performed by the enzyme procedure of Finch *et al.*[37].

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