Enzymatic synthesis of a blood group A related, difucosyl heptaglycosylceramide with a type 2 carbohydrate chain

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The presence of an $\alpha 1 \longrightarrow 3$ fucosyltransferase in the serum of an A_1Rh(−) Le(a−b+) Fy(a−b+) Jk(a+b+) K(−) M(−) N(+) S(−) s(+) P_1(+) human secretor was shown, capable of converting a blood group A active hexaglycosylceramide with a type 2 carbohydrate chain into the corresponding difucosyl compound under in vitro conditions.

Glycolipid *Fucolipid* *Blood group A* *Glycosyltransferase* *Fucosyltransferase* *GDP-fucose*

1. INTRODUCTION

The carbohydrate structures in glycolipids, glycoproteins and oligosaccharides that confer A, B, H and Lewis blood group specificities on these molecules are built up by sequential action of a series of glycosyltransferases determined by genes at several different loci. The blood group genes at the ABO, H and Lewis loci are concerned only in the final stages of the biosynthesis of the carbohydrate structures [1]. The blood group A active structures terminate with *N*-acetyl-D-galactosamine in an α-linkage to the C-3 position of a subterminal β-D-galactosyl unit which is also substituted at the C-2 position with L-fucose by the blood group H glycosyltransferase. In these oligosaccharides the β-D-galactosyl residues may be joined [1] by either a $1 \longrightarrow 3$ (type 1 chain) or a $1 \longrightarrow 4$ (type 2 chain) linkage to the following *N*-acetyl-D-glucosamine (table 1).

The Lewis gene controls a glycosyltransferase which adds L-fucose on the 4 position of the *N*-acetyl-D-glucosamine and therefore the Le^a and Le^b active structures are based only on type 1 chains. The corresponding type 2 chain isomers have also been isolated but lack serologic Le^a or Le^b activity. The gene that controls a glycosyltransferase which adds L-fucose on the 3 position of the *N*-acetyl-D-glucosamine in these compounds appears to have a high frequency [2–4]. One unique exception has however been described [5]. Carbohydrate determinants related to blood group A, but with two fucosyl substituents, have been isolated from ovarial cyst glycoproteins and human urine oligosaccharides [6,7] and important data concerning their biosynthesis have been presented [8,9]. Blood group A difucosyl heptaglycosylceramides (glycolipid) have been identified and characterized from various sources [10] and nothing is so far known about their biosynthesis. The aim of the present investigation was to study the biosynthesis of these compounds.

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2. MATERIALS AND METHODS

Serum was obtained from an A_1Rh(−) Le(a−b+) Fy(a−b+) Jk(a+b+) K(−) M(−) N(+) S(−) s(+) P_1(+) secretor and concentrated 4 times using a 'Minicon' concentrator at +4°C.

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Table 1

Some carbohydrate chain endings of relevance to the text

GalNAcα1 → 3Galβ1 → 3GlcNAc- 2 ↑ Fucα1	Blood-group A determinant type 1 chain
GalNAcα1 → 3Galβ1 → 4GlcNAc- 2 ↑ Fucα1	Blood-group A determinant type 2 chain
Galβ1 → 3GlcNAc- 4 ↑ Fucα1	Le^a determinant type 1 chain
Galβ1 → 4GlcNAc- 3 ↑ Fucα1	X determinant type 2 chain [23]
Galβ1 → 3GlcNAc- 2 4 ↑ ↑ Fucα1 Fucα1	Le^b determinant type 1 chain
Galβ1 → 4GlcNAc- 2 3 ↑ ↑ Fucα1 Fucα1	Y determinant type 2 chain [23]
GalNAcα1 → 3Galβ1 → 3GlcNAc- 2 4 ↑ ↑ Fucα1 Fucα1	Blood-group A, difucosyl determinant type 1 chain
GalNAcα1 → 3Galβ1 → 4GlcNAc- 2 3 ↑ ↑ Fucα1 Fucα1	Blood-group A, difucosyl determinant type 2 chain

The concentrated serum sample was used immediately or stored at −20°C before use. The serum preparation was incubated under essentially the same conditions as were described [4]. The incubation mixture contained in a final volume of 100 μl: 60 μl of the serum preparation, about 130000 cpm of GDP-[^{14}C]fucose (Radiochemical Centre, Amersham), 2.5 μmol Tris–HCl buffer (pH 7.5), 1 μmol NaN_3, 0.75 μmol $MgCl_2$, 1 μmol ATP, Triton X-100 0.1% and precursor glycolipid 50–200 μg. The incubation mixture was kept at 37°C for 48 h under continuous gentle agitation.

The incubation mixture was extracted sequentially by 5 ml of methanol, chloroform/methanol 2:1 v/v, chloroform/methanol 1:2 v/v and methanol at 70°C. The combined extracts were evaporated to dryness and subjected to mild alkaline hydrolysis and dialysis. Non-acid glycosphingolipids were prepared by a combination of silicic acid chromatography and DEAE chromatography essentially as described [11].

The total non-acid glycosphingolipid fraction prepared from the incubation mixtures was analysed by thin-layer chromatography as native and acetylated derivatives.

Thin-layer chromatography was performed on HPTLC plates (Merck). For autoradiographic detection Ilford 25GP X-ray film was used. After exposure the film was developed using Ilford Phen-X, X-ray developer. Determination of radioactivity was also done of an aliquot of the fraction in a liquid scintillation spectrometer (Packard) after evaporation of the organic solvent and addition of 10 ml of a solution containing 3 g PPO, 75 mg POPOP, 500 ml toluene and 250 ml Triton X-100. For detection of non-radioactive glycolipids the anisaldehyde reagent was used [12]. The blood group A active hexaglycosylceramide

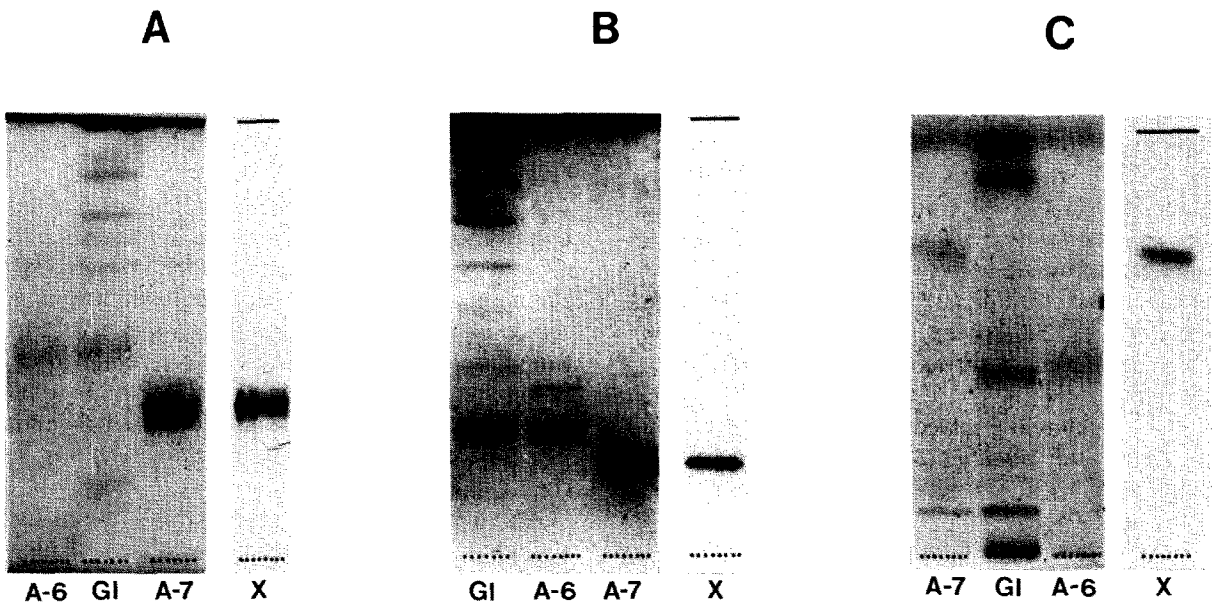

Fig.1. Thin-layer chromatograms showing the non-acid glycosphingolipid fraction Gℓ, prepared from an incubation mixture that contained a blood group A active hexaglycosylceramide with a type 2 carbohydrate chain isolated from dog intestine, GDP-[^{14}C]fucose and serum from an A_1Rh(−) Le(a−b+) secretor, analysed as native derivatives in two different solvents, fig.1A and B, and as acetylated derivatives in one solvent, fig.1C. The precursor glycolipid, A-6 (blood group A active hexaglycosylceramide with a type 2 chain), and the expected product, A-7 (blood group A similar difucosyl heptaglycosylceramide with a type 2 carbohydrate chain isolated from dog intestine) are also analysed as references. The solvent systems for the thin-layer plates were as follows: (A) chloroform/methanol/NH_3 (conc.), 40:40:12, by vol.; (B) chloroform/methanol/water, 60:35:8, by vol.; (C) chloroform/methanol, 95:5, by vol. After development the thin-layer plates were covered with an X-ray film and left in the dark for 4 days. Lane X in A–C above shows the result of the autoradiographic detection of lane Gℓ in each case. After autoradiography the plates were visualized with the anisaldehyde reagent [12].

used as a precursor and the reference heptaglycosylceramide were both isolated from dog intestine and shown to have the following structures: GalNAcα1 ⟶ 3Gal(2 ⟵ 1αFuc)β1 ⟶ 4GlcNAcβ1 ⟶ 3Galβ1 ⟶ 4Glcβ1 ⟶ 1Ceramide [13,14] and GalNAcα1 ⟶ 3Gal(2 ⟵ 1αFuc)β1 ⟶ 4GlcNAc(3 ⟵ 1αFuc)β1 ⟶ 3Galβ1 ⟶ 4Glcβ1 ⟶ 1Ceramide ([15,16] and McKibbin et al. unpublished). The ceramide composition was hydroxy fatty acids in combination with about equal amounts of the sphingosine bases t18:0 and d18:1. The ceramide composition was almost identical between the two glycolipids.

3. RESULTS

Figure 1A–C show the thin-layer chromatogram of the non-acid glycosphingolipid fraction, Gℓ, prepared from an incubation mixture that contained a blood group A active hexaglycosylceramide with a type 2 carbohydrate chain isolated from dog intestine, GDP-[^{14}C]fucose and serum from an A_1Rh(−) Le(a − b +) secretor, analysed as native derivatives in two different solvents, fig.1A and B, and as acetylated derivatives in one solvent, fig.1C. The precursor glycolipid, A-6 (blood group A active hexaglycosylceramide, type 2 chain), and the expected product, A-7 (the corresponding heptaglycosylceramide with two fucose residues and a terminal hexosamine, type 2 chain), are also analysed as references. Lane X shows the autoradiographic detection of lane Gℓ of the thin-layer plate.

The non-acid glycosphingolipid fraction prepared after incubation, Gℓ, contained 2400 counts/min (130000 cpm GDP-[^{14}C]fucose was added initially).

As can be seen the radioactive spot on the autoradiogram has the same R_f-value as the reference glycolipid, A-7, in all solvents used, both as native and as acetylated derivatives.

4. DISCUSSION

The preparation of a pure non-acid glycosphingolipid fraction from the incubation mixture is considered important for the interpretation of results. An autoradiogram is obtainable at any preparative stage but the probability of a glycolipid causing the radioactive spot increases with purity. This concept is not always appreciated.

Identification of the product was based on comparison of the thin-layer chromatographic mobility with a known reference of the expected product. The comparison was made both as native substances and as acetylated derivatives and using different solvent systems. Acetylation of glycosphingolipids has a profound effect on their chromatographic mobility and may also change the order between individual glycosphingolipids [17,18].

The ceramide composition of glycosphingolipids is also known to influence the chromatographic mobility. The partial separation into minor bands on the thin-layer plates, figs.1A–C, is most probably caused by a heterogeneous ceramide as the carbohydrate part is uniform [13–16]. It is therefore considered important to use reference compounds of similar ceramide compositions as the expected product for a safe identification based on thin-layer chromatographic mobility. In the present case both the precursor glycolipid and the reference glycolipid were prepared from the same source and shown to have almost identical ceramide composition [13–16].

Although no structural characterization (mass spectrometry, NMR, degradation) was done on the radioactive product, it appears safe to conclude the presence of an α1 ⟶ 3 fucosyltransferase in the serum of a human A, Le(a − b +) secretor (additional typing, see above), capable of converting a blood group A active hexaglycosylceramide with a type 2 carbohydrate chain into the corresponding difucosyl compound under in vitro conditions, see fig.2.

An alternative pathway would be from an Le^b-similar hexaglycosylceramide with type 2 chain (table 1). However, a highly purified porcine blood group A specific *N*-acetylgalactosaminyltransferase [9] failed to work on an Le^b-active hexaglycosylceramide (type 1 chain). Unfortunately, the type 2 chain isomer was not tested.

The present knowledge about Fucα1 ⟶ 3 transferase(s) is complex. Recently [19] Fucα1 ⟶ 3 and Fucα1 ⟶ 4 transferases were co-purified over 500000-fold from human milk, suggesting that both enzymatic activities reside in a single enzyme and thus obscuring the Lewis genetics (Le gene codes for a Fucα1 ⟶ 4 transferase). Further-

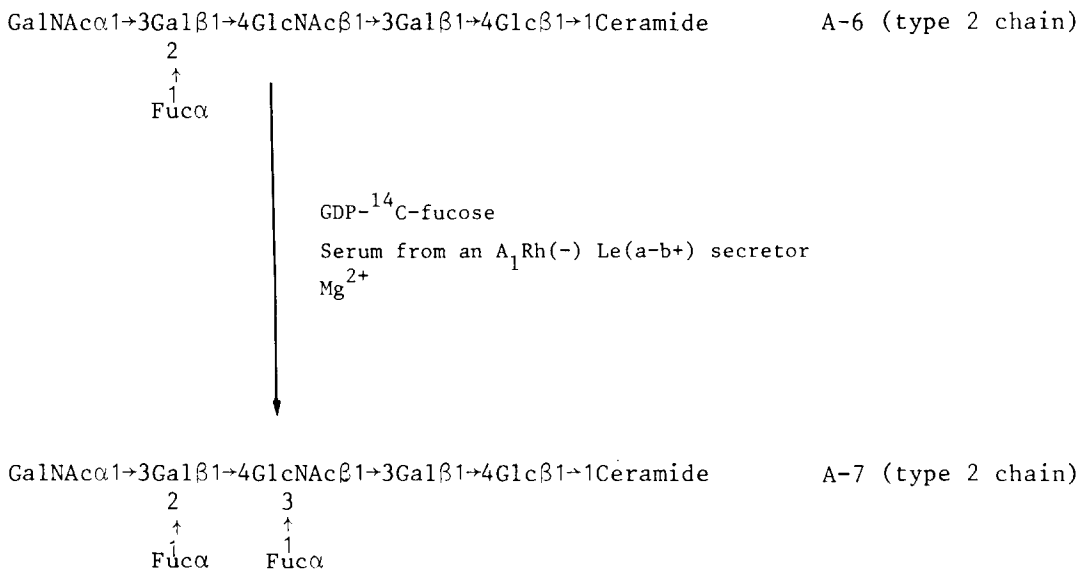

Fig.2. Proposed biosynthetic scheme.

more, evidence has been presented for the presence of two Fucα1 ⟶ 3 transferases in human saliva [20]. Both enzymes were shown capable of transferring L-fucose to the C-3 position of *N*-acetyl-D-glucosamine while only one had capacity of transferring L-fucose to C-3 of D-glucose and was furthermore dependent on the Le gene. If the enzymatic activity described in this paper is due to an additional enzyme species in a Fucα1 ⟶ 3 transferase family or is due to a common Fucα1 ⟶ 3 transferase with a limited precursor specificity requires further studies.

The biosynthesized difucosyl heptaglycosylceramide (type 2 chain) has so far not been found on red blood cell membranes [21]. Its type 1 chain isomer, the Siedler antigen, has however been shown to be taken up on red cells from plasma [22]. The site for its biosynthesis is unknown. The biological importance of the described Fucα1 ⟶ 3 enzymatic activity remains to be elucidated.

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