IMMUNOCHEMISTRY OF THE LEWIS BLOOD-GROUP SYSTEM

Investigations on the Le^c antigen

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1. Introduction

The Lewis blood-group system comprises 5 antigens – Le^a, Le^b, Le^c, Le^d and Le^x [19]. In investigations on the molecular structures of different blood-group Lewis active glycosphingolipids from human plasma and red cells, the Le^a, Le^b and Le^{dH} antigens could be identified as being lacto-*N*-fucopentaosyl(II)-ceramide, lacto-*N*-difucohexaosyl(I)ceramide and lacto-*N*-fucopentaosyl(I)ceramide, respectively [1–5]. The Le^x property is probably based on a particular specificity of the anti-Le^x antibodies rather than a distinct antigenic determinant; it has been shown that anti-Le^x binds to the Lewis core disaccharide Fucα1→4GlcNAc-R [8], a property which enables the antibody to react with both Le^a and Le^b determinants. This specificity may render the antibody less sensitive to steric effects, and thus able to bind even to the traces of Le^a substance present on cord red cells. As Le^a of Lewis-positive non-secretors can be regarded at least formally as the precursor of Le^b in Lewis-positive secretors, and since it is now well-established that the Le^{dH} antigen of Le(a–b–) secretors can be referred to H type 1 glycosphingolipid [3–5], it would be a matter of logical consequence to suggest that the Le^c antigen detected on the red cells of Le(a–b–) non-secretors might be lacto-*N*-tetraosylceramide, the precursor of the H type 1 (Le^{dH}) antigen (see also [6]). In contrast to this consideration, based on a weak inhibition capacity of 3′-fucosyllactose, Watkins proposed the structure Galβ1→4GlcNAc3←1αFuc for the Le^c determinant (see [9]). This result was recently confirmed by similar inhibition studies [17].

This paper describes our latest immunochemical experiments on the Le^c antigenicity and represents a critical re-examination of these hypotheses.

Abbreviations and trivial names: Glc, glucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Fuc, fucose; Cer, ceramide; lacto-*N*-tetraosylceramide, Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ1→1 Cer; lacto-*N*-neotetraosylceramide, Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1 Cer; H type 1 (Le^{dH}), blood-group active lacto-*N*-fucopentaosyl(I)ceramide, Fucα1→2Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ1→1 Cer; Le^a blood-group active lacto-*N*-fucopentaosyl(II)ceramide, Galβ1→3GlcNAc-(4←1αFuc)β1→3Galβ1→4Glcβ1→1 Cer; lacto-*N*-fucopentaosyl-(III)ceramide, Galβ1→4GlcNAc(3←1αFuc)β1→3Galβ1→4Glcβ1→1 Cer; 3′-fucosyllactose, Galβ1→4Glc3←1αFuc; apoHDL, delipidated human high-density lipoprotein; HPTLC, high-performance thin-layer chromatography

This paper is the seventh of a series on the immunochemistry of the blood-group Lewis system [1–5,8]

2. Materials and methods

2.1. *Glycosphingolipids*

Lacto-*N*-neotetraosylceramide was prepared from human erythrocyte membranes as in [10]. It was also obtained from rabbit erythrocyte ceramide pentasaccharide by α-galactosidase treatment [11,12]. H type 1 (Le^{dH}) blood-group active lacto-*N*-fucopentaosyl(I)-ceramide was isolated, purified and identified as in [3–5]. It was degraded to lacto-*N*-tetraosylceramide by mild acid hydrolysis with 0.1 M trichloracetic acid at 100°C for 2 h. Purification was performed by preparative silica gel HPTLC [1]. Le^a blood-group active lacto-*N*-fucopentaosyl(II)ceramide was isolated from human plasma as described previously.

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2.2. *Enzymes*

A mixture of α1→3 and Lewis-gene-dependent α1→4 fucosyltransferases was obtained from human milk by ammonium sulphate fractionation and CM-cellulose chromatography as in [14]. Two different preparations (I and II) were used here.

α1→3/4 and α1→2 fucosidase were purified from an ultrasonic extract of *Trichomonas foetus* by fractionation on Sepharose 4B followed by chromatography on DEAE-cellulose [13], the enzymes being eluted with 0.7 M and 0.15 M KCl, respectively.

2.3. *Biosynthesis of glycosphingolipids*

GDP-fucose was prepared as in [15]. Delipidated human high density lipoprotein (apoHDL) was a generous gift from Professor Gerd Assmann (Zentrallabor und Abt. für klinische Chemie, University of Münster).

Lactotetraosylceramide (100 μg) or neolactotetraosylceramide (100 μg) recombined with apoHDL as in [12] and dialyzed against 50 mM Tris–HCl buffer (pH 7.0) for 4 h, were incubated for 46 h at 37°C with the α1→3/α1→4 fucosyltransferase preparation (15 mg protein) in the presence of 2 μmol $MgCl_2$ and 0.2 μmol GDP-fucose and mixed continuously with a magnetic stirring bar, the total volume being 300 μl. The extraction of the glycolipids was performed as described in the following section.

2.4. *Enzymatic degradation*

Fucolipid (100 μg) was recombined with 200 μg apoHDL as in [12]. The resulting glycolipid–lipoprotein complex was incubated with different fucosidases for 18 h at 37°C in 50 mM Tris–HCl buffer (pH 7.0). Subsequently 4 vol. chloroform/methanol (2/1, v/v) were added, and the mixture was vigorously agitated. After centrifugation and removal of the lower phase, the remaining upper phase was extracted again with theoretical lower phase. The combined lower phases were evaporated to dryness and used for HPTLC and immunological investigations.

2.5. *Antisera*

Anti-Le^a sera and anti-Le^b sera were purchased from Ortho Diagnostic Systems, Raritan (goat), Behring, Marburg (goat), and Molter, Heidelberg (human). Human anti-Le^c was obtained from the blood donor 'ARM' [9]. Animal anti-Le^c was prepared by immunization of goats as in [16], using blood-group substances from boiled saliva of Le(a–b–) non-secretors as immunogen [6].

2.6. *Immunological methods*

Haemagglutination inhibition and passive haemagglutination tests were performed as in [1], except that for haemagglutination inhibition tests using anti-Le^c 'ARM', erythrocytes from O Le(a–b–) non-secretors were treated with papain. Incubation of packed erythrocytes (100 μl) in 500 μl phosphate-buffered saline (pH 7.4) containing 0.01% papain (Merck, Darmstadt) for 5 min at 37°C, was determined to yield a maximum agglutination strength of Le(a–b–) cells from non-secretors without simultaneous agglutination of erythrocytes of all other Lewis and secretor phenotypes.

3. Results and discussion

To investigate whether the antigenic determinant of the Le^c property might reside in type 2 chain glycolipids, haemagglutination inhibition and passive haemagglutination tests were performed with lacto-*N*-fucopentaosyl(III)ceramide and its precursor, lacto-*N*-neotetraosylceramide. The lacto-*N*-fucopentaosyl(III)-ceramide was prepared by enzymatic transfer of fucose onto lacto-*N*-neotetraosylceramide. In fig.1, lanes 8 and 9 demonstrate the results of the incubation of lacto-*N*-neotetraosylceramide with 2 different preparations of α1→3/α1→4 fucosyltransferase of human milk in the presence of GDP-fucose. In both cases a glycolipid was produced which had a HPTLC migration behaviour very similar to, but definitely distinct from that of the Le^a-I glycolipid (nomenclature in [1–3]). These glycolipid fractions, as well as their precursor, lacto-*N*-neotetraosylceramide, failed to show even partial inhibition of 4 haemagglutinating units of anti-Le^c 'ARM' or goat anti-Le^c, 50 μg substance being used for each assay. Furthermore, coating of 9×10^7 O Le(a–b+) erythrocytes with 50 μg of these glycolipids did not render the cells agglutinable by either of the 2 Le^c antisera.

These results provide good evidence that the 3′-fucosyllactosamine as well as its corresponding type 2 chain precursor, *N*-acetyllactosamine, can be excluded as a possible molecular basis of the Le^c antigen.

To investigate the hypothesis that type 1 chain glycolipids are involved in the Le^c antigenicity, the following experiments were performed: after mild acid hydrolysis of the H type 1 (Le^{dH}) blood-group active lacto-*N*-fucopentaosyl(I)ceramide (Le^{dH}-I, fig.1, lane 1) a degradation product was obtained which

showed a slightly faster migration behaviour than lacto-*N*-neotetraosylceramide (fig.1, lane 3). This substance, regarded as lacto-*N*-tetraosylceramide, was also investigated for its ability to inhibit the anti-Le^c sera. Using 4 haemagglutinating units of either anti-Le^c 'ARM' or goat anti-Le^c and erythrocytes from an Le(a−b−c+) donor, no complete or incomplete inhibition of agglutination by 30 μg glycolipid was achieved with either antiserum. Likewise tests of quantitative passive haemagglutination failed to reveal any Le^c activity of the degradation product. These results are not in accordance with [18]: upon immunization of rabbits with Galβ1→3GlcNAc coupled to bovine serum albumin they obtained an antiserum which after purification by appropriate affinity columns agglutinated the erythrocytes of a Le(a−b−) non-secretor but not those from Le(a+b−), Le(a−b+), or Le(a−b−) secretor individuals. This apparent contradiction led us to consider that our substance prepared from H type 1 (Le^{dH}) glycolipid might be

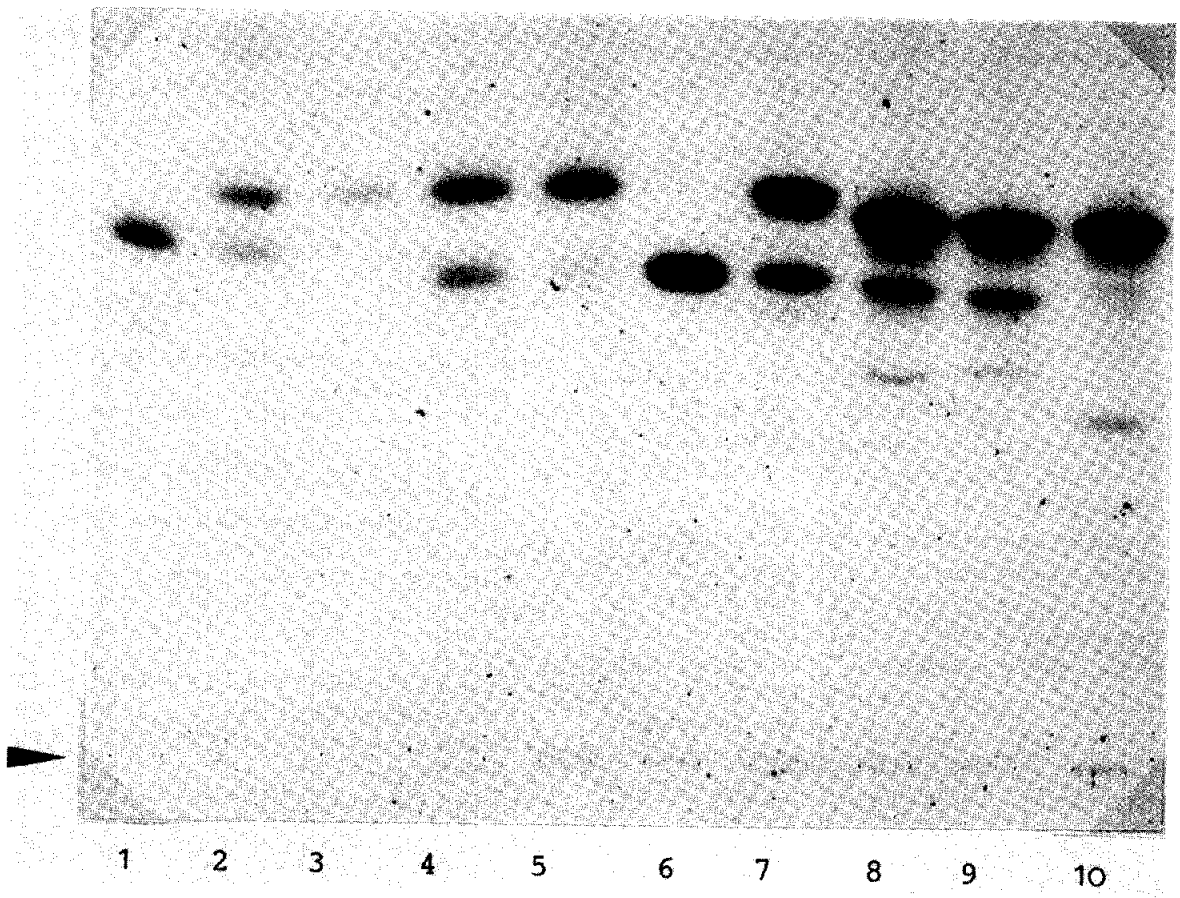

Fig.1. High-performance thin-layer chromatography of glycosphingolipid fractions obtained after incubation of lacto-*N*-fucopentaosyl(I)ceramide (H type 1 (Le^{dH})), lacto-*N*-tetraosylceramide (LNT-Cer), lacto-*N*-fucopentaosyl(II)ceramide (Le^a), and lacto-*N*-neotetraosylceramide (LNneoT-Cer) with different fucosidases or fucosyltransferases: (1) H type 1 (Le^{dH}); (2) H type 1 (Le^{dH}) after treatment with α1→2 fucosidase in the presence of apoHDL; (3) LNT-Cer; (4) Le^a after treatment with α1→4 fucosidase in the presence of apoHDL; (5) LNT-Cer; (6) Le^a; (7) LNT-Cer after incubation with α1→3/α1→4 fucosyltransferase (preparation I) in the presence of apoHDL and GDP-fucose; (8) LNneoT-Cer after incubation with α1→3/α1→4 fucosyltransferase (preparation I) in the presence of apoHDL and GDP-fucose; (9) LNneoT-Cer after incubation with α1→3/α1→4 fucosyltransferase (preparation II) in the presence of apoHDL and GDP-fucose; (10) LNneoT-Cer (not completely purified).

Abbreviations: lacto-*N*-tetraosylceramide, Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ1→1 Cer; lacto-*N*-neotetraosylceramide: Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1 Cer; lacto-*N*-fucopentaosyl(II)ceramide (Le^a), Galβ1→3GlcNAc(4←1αFuc)β1→3Galβ1→4Glcβ1→1 Cer; H type 1 (Le^{dH}), Fucα1→2Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ1→1 Cer

misidentified or, an artifact. The structure of the degradation product was therefore checked by a number of methods:

(1) 360 MHz ^{1}H NMR spectroscopical analysis yielded spectra, with a signal pattern in complete accord with the structure of a lacto-*N*-tetraosylceramide [5]. In addition, data obtained by spin decoupling difference spectroscopy and nuclear Overhouser enhancement represent direct proof of the above structure [5]. The resonances obtained showed no evidence of contamination by impurities [5].

(2) Treatment of Le^{dH}-I with $\alpha 1 \rightarrow 2$ fucosidase from *Trichomonas foetus* yielded a glycolipid showing a migration behaviour identical to that of the degradation product obtained by chemical hydrolysis (fig.1, lane 2 and 3 respectively).

(3) Degradation of Le^a substance (Le^a-I glycolipid) with $\alpha 1 \rightarrow 4$ fucosidase from *Trichomonas foetus* yielded a glycolipid with a migration behaviour which was also identical to that obtained by chemical or enzymatic degradation of Le^{dH}-I (fig.1, lane 4).

(4) Furthermore, Le^{dH}-I pre-degraded by acid hydrolysis and subsequently incubated with the Le-gene dependent $\alpha 1 \rightarrow 4$ fucosyl-transferase yielded a Le^a blood-group active glycolipid which had an R_F-value identical to that of Le^a-I (fig.1, lane 7).

These experiments unambiguously show, that the substance obtained from Le^{dH}-I by chemical degradation is lacto-*N*-tetraosylceramide and that also this glycolipid does not represent the Le^c antigen.

Considering the investigations in [18] it seems most reasonable to propose that the Le^c antigen has a type 1 oligosaccharide core with at least one additional sugar unit linked to the lacto-*N*-tetraosylceramide. Identification studies, i.e., purification and structural elucidation of the Le^c glycolipid are now in progress.

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