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Improved procedure for the construction of neoglycolipids having antigenic and lectin-binding activities, from reducing oligosaccharides

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Conditions have been established for the rapid and efficient conjugation of reducing oligosaccharides (di- to deca-saccharides) to dipalmitoyl phosphatidylethanolamine. The resulting neoglycolipids derived from several naturally occurring oligosaccharides and a series of *N*-linked high-mannose-type oligosaccharides released by hydrazinolysis from RNAase B showed specific and potent reactivities, as appropriate, with monoclonal antibodies to blood group Lewis^b, blood group A or a stage-specific embryonic (SSEA-1) antigen, or the lectin concanavalin A.

INTRODUCTION

As part of our programme of research on oligosaccharide recognition by antibodies and diverse other carbohydrate-binding proteins, we (Tang *et al.*, 1985) established micro procedures involving the construction of oligosaccharide probes, i.e. neoglycolipids derived from single oligosaccharides, mixtures of oligosaccharides or their alditols by reductive amination with dipalmitoyl phosphatidylethanolamine (PPEADP). Unlike oligosaccharides, which are hydrophilic molecules, the neoglycolipids have hydrophobic properties conferred by the lipid moiety, such that ligand-binding assays can be performed by using the oligosaccharides as immobilized probes on silica gel and plastic plates, or as microparticulate probes in liposomes. In each case the clustering achieved resulted in an enhancement of the reactivities of the oligosaccharides with monoclonal antibodies. In the course of detailed analyses of reaction products under different conjugation conditions, we have observed that the water content of the reaction mixture has a marked influence on the conjugation rate of reducing oligosaccharides. We report here (*a*) conditions for efficient conjugation of several natural free oligosaccharides (di- to hepta-saccharides) and a series of *N*-linked oligosaccharides (hepta- to deca-saccharides) of high-mannose type released from protein by hydrazinolysis and (*b*) the potent and specific reactivities of the resulting neoglycolipids in oligosaccharide-recognition assays on silica-gel chromatograms.

MATERIALS AND METHODS

Materials

High-performance t.l.c. plates (aluminium-backed silica, 5 μm thick; Merck) were from BDH, Poole, Dorset, U.K. Bovine pancreatic RNAase B, concanavalin A, lactose, sodium cyanoborohydride and PPEADP were from Sigma, Poole, Dorset, U.K. Plexigum P28 (Röhm) was from Cornelius Chemical Co., Romford, Essex, U.K. Other chemicals were Analytical Reagent grade.

Oligosaccharides

The human milk oligosaccharides lacto-*N*-fucopentaose III [Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc] and lacto-*N*-difucohexaose I [Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc] and the blood-group 'A-like' disaccharide GalNAcα1-3Gal prepared from partial acid hydrolysis products of an ovarian-cyst glycoprotein (Coté & Morgan, 1956) were gifts from Dr. W. M. Watkins and Dr. A. S. R. Donald. The blood-group A tetrasaccharide GalNAcα1-3(Fucα1-2)Galβ1-4Glc, isolated from human urine (Strecker *et al.*, 1973), was a gift from Dr. G. Strecker; blood-group A hexasaccharide GalNAcα1-3(Fucα1-2)Galβ1-3GlcNAcβ1-3Galβ1-4Glc and blood-group A heptasaccharide GalNAcα1-3(Fucα1-2)Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc, isolated from faeces of breast-fed children (Sabharwal *et al.*, 1983), were gifts from Dr. A. Lundblad and Dr. H. Sabharwal. A mixture of the oligosaccharides $Man_5GlcNAc_2$, $Man_6GlcNAc_2$, $Man_7GlcNAc_2$ and $Man_8GlcNAc_2$, with the sequence (Manα1-2)$_{0-3}$-(Manα1-6)(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc, was obtained from RNAase B (Liang *et al.*, 1980) by hydrazinolysis for 10 h (Takasaki *et al.*, 1982).

Mouse hybridoma antibodies

Antibody 115H10 directed at the mammary milk-fat-globule antigen MAM-3b (Hilkens *et al.*, 1984) and specific for the blood-group Le^b-related oligosaccharide sequence Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Gal (Gooi *et al.*, 1985*a*) was a gift from Dr. J. Hilkens and Dr. J. Hilgers. Anti-SSEA-1, directed at the stage-specific embryonic antigen SSEA-1 (Solter & Knowles, 1978) and specific for the oligosaccharide sequence Galβ1-4(Fucα1-3)GlcNAc (Gooi *et al.*, 1981) was a gift from

Abbreviations used: BSA, bovine serum albumin: PBS, phosphate-buffered saline; PPEADP, dipalmitoyl phosphatidylethanolamine; TBS, 20 mM-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl, 10 mM-$CaCl_2$ and 1 mM-$MnCl_2$.

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Dr. D. Solter. Antibody MH-1, raised against a human colon-cancer cell line (D. Voak & E. S. Lennox, unpublished work) and specific for mono- and di-fucosyl blood-group A structures on either Type 1 or Type 2 backbones, GalNAcα1-3(Fucα1-2)Galβ1-3/4(±Fucα1-4/3)Glc/GlcNAc (Gooi *et al.*, 1985*b*) was a gift from Dr. E. S. Lennox. Antibody TH-1, raised against blood-group A_1 erythrocytes and directed against the repetitive blood-group A sequence GalNAcα1-3(Fucα1-2)Galβ1-3GalNAcα1-3(Fucα1-2)Galβ1- (Clausen *et al.*, 1985), was a gift from Dr. S. Hakomori. Antibody NCC-LU-35, raised against membranes of human lung-cancer cells and directed against GalNAcα1- (Hirohashi *et al.*, 1985), was a gift from Dr. S. Hirohashi. The antibodies were used as undiluted culture supernatant (MH-1, TH-1 and LU-35) or as 1:100 (v/v) dilutions of ascites (115H10 and anti-SSEA-1). Normal mouse serum (1:100 dilution) or tissue-culture medium containing 10% (v/v) fetal-calf serum was used as a negative control.

Construction of neoglycolipids

The procedure was adapted from that described by Tang *et al.* (1985). Lactose (500 μg; 1.46 μmol) was evaporated to dryness under N_2 and mixed with 7.65 mg of PPEADP (11.9 μmol) in 1.53 ml of chloroform/methanol (1:1, v/v) containing various amounts of water [0, 0.5, 1, 2, 3, 5 and 10% (v/v)]. The mixture was sonicated (10 min) in a sonic bath (Dawe Sonicleaner; Dawe Instruments, Branson Electronics, Danbury, CT, U.S.A.) and heated (50 °C, 2 h) in reaction vials sealed with Teflon-lined caps. Then 350 μg of sodium cyanoborohydride (5.55 μmol) in methanol (35 μl) was added and the mixture was again heated (50 °C). Reaction mixtures derived from 2 μg of lactose were withdrawn at intervals during 16 to 188 h for immediate analysis by t.l.c. The extent of derivative formation was estimated by colour matching of residual lactose with a series of lactose standards stained with orcinol reagent. Experiments were performed in duplicate. Unless otherwise specified, other oligosaccharides were conjugated under the anhydrous condition with incubation at 50 °C for 16 h after the addition of sodium cyanoborohydride. Before chromatography of anhydrous reaction mixtures containing trisaccharides and larger oligosaccharides, 10% (v/v) water was added. For conjugation of the oligosaccharides from RNAase B, 200 μg of the oligosaccharide mixture was dissolved in 20 μl of water and mixed with 1.9 mg of PPEADP dissolved in 380 μl of chloroform/methanol (1:1, v/v). After incubation at 60 °C for 2 h, 0.4 mg of sodium cyanoborohydride in 40 μl of methanol was added, and the reaction was continued for 16 h at 60 °C.

T.l.c.

For t.l.c. the following solvent systems were used: chloroform/methanol/water (11:9:2, by vol.) (solvent 1) or chloroform/methanol/water (105:100:28, by vol.) (solvent 2).

Binding of antibodies and lectin to neoglycolipids on t.l.c. plates

For chromatogram binding assays (Magnani *et al.*, 1987), dried chromatograms were soaked for 30 s in n-hexane containing 0.1% (w/v) Plexigum P28, dried and soaked for 2 h in phosphate-buffered saline (PBS; Dulbecco's A) containing 3% (w/v) bovine serum albumin (BSA). Strips were drained and overlaid with antibodies at 4 °C for 16 h. Strips were washed with PBS and overlaid with PBS containing 1% (w/v) BSA and 5×10^5 c.p.m. of ^{125}I-labelled rabbit antibodies to mouse immunoglobulins (Dako, Copenhagen, Denmark)/ml. For binding assays with ^{125}I-labelled concanavalin A, 5×10^5 c.p.m./ml was used, and PBS was replaced with 20 mM-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl, 10 mM-$CaCl_2$ and 1 mM-$MnCl_2$ (TBS), with or without added 200 mM-α-methyl mannoside. After 2 h, strips were washed with PBS or TBS and dried. Autoradiography was performed at −70 °C for 16–108 h by using Kodak type S film and a Philips ultra-S intensifying screen.

RESULTS

Preliminary experiments showed that, by using solvent 1, the lactose–PPEADP conjugate was effectively separated from the free disaccharide and free lipid (Fig. 1, panel 1), and that water content and total reaction volume greatly affected the extent of conjugation: at 16 h conjugation was near complete in the anhydrous condition, but was retarded in the presence of 10% (v/v) water or if the reaction volume was increased 5-fold (Fig. 1, panel 2). In subsequent time-course experiments (Fig. 2) it was found that the conjugation rate progressively decreased with increasing water content. In experiments with several antigenic oligosaccharides (di-

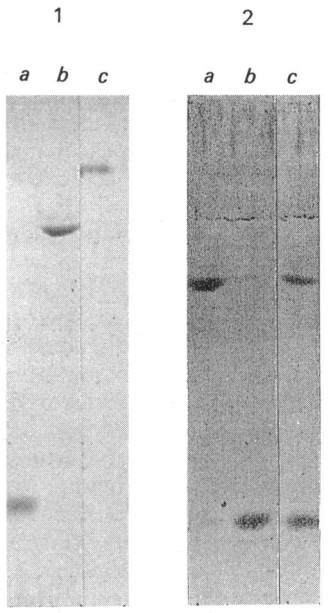

Fig. 1. Thin-layer chromatograms of lactose, PPEADP and their conjugates

Panel 1 shows separations of free lactose (lane *a*), lactose–PPEADP conjugate (lane *b*) and free PPEADP (lane *c*). Panel 2 shows the reaction mixtures after conjugation of lactose to PPEADP at 16 h under three conditions: anhydrous condition as defined in the Materials and methods section and reaction volume 1.53 ml (lane *a*); water content 11.5% (v/v) and reaction volume as before (lane *b*); anhydrous condition and reaction volume 7.65 ml (lane *c*). Total lactose applied was 1 μg/lane; chromatography was in solvent 1, staining was with orcinol reagent, except for lane *c* in panel 1, with ninhydrin reagent. Arrow indicates point of application.

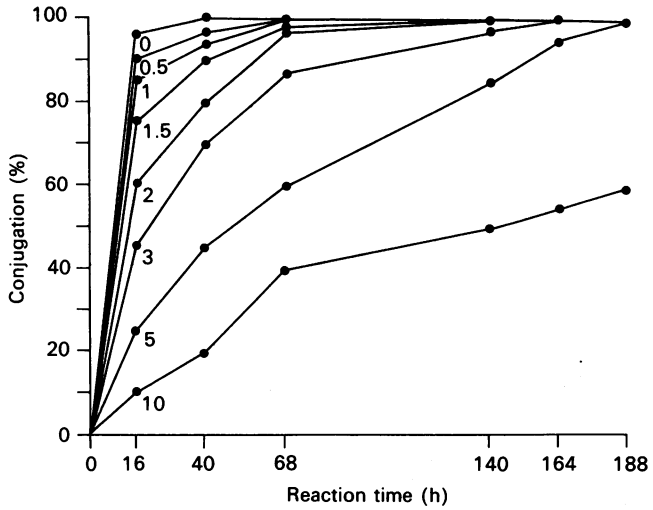

Fig. 2. Time-course experiments showing the extent of conjugation of lactose to PPEADP in the presence of different amounts of water (0–10%, v/v)

Reaction volume was 1.53 ml; reaction mixtures (derived from 2 μg of lactose) were chromatographed in solvent 1 and stained with orcinol reagent. The amount of non-conjugated lactose was estimated by colour matching with lactose standards. Numbers on curves indicate water content (%, v/v).

to hepta-saccharides) conjugation again occurred more rapidly under anhydrous conditions (results not shown).

The specificity of antibody binding to the neoglycolipids is shown in Fig. 3: when the neoglycolipids of the two fuco-oligosaccharides, lacto-*N*-fucopentaose III and lacto-*N*-difucohexaose I, were chromatographed as a mixture, anti-SSEA-1 reacted only with the pentaose, whereas anti-MAM-3b reacted only with the hexaose (Fig. 3, panel 1). This is in accordance with the known specificities (Gooi *et al.*, 1981, 1985*a*) of these monoclonal antibodies. With antibody LU-35 all three blood-group-A-related oligosaccharides, di-, tetra- and hexa-saccharide, gave positive immunostaining (Fig. 3, panel 2), in accordance with the known specificity of this antibody for the GalNAcα1 structure (Hirohashi *et al.*, 1985). With antibody MH-1, the blood-group A tetra-, hexa- and hepta-saccharide gave positive immunostaining (Fig. 3, panels 2 and 3), in accordance with the broad specificity of this antibody for the mono- and di-fucosyl blood-group A structures based on type 1 (Galβ1-3Glc/GlcNAc) or type 2 (Galβ1-4Glc/GlcNAc) backbones. Antibody TH-1, which has been reported to have a specificity for the repetitive blood-group A sequence (Clausen *et al.*, 1985), did not react with the blood-group A di-, tetra- and hepta-saccharide, but it appeared to react with a minor component in the A hexasaccharide which chromatographed close to the major orcinol-stained component in solvent 1 (Fig. 3, panel 2) and at its leading edge in solvent 2 (Fig. 3, panel 3). This reactivity is most likely to be due to the presence of a trace contaminant in the A hexasaccharide preparation, since the repetitive blood-group A sequence could not be detected by n.m.r. (E. F. Hounsell, unpublished work).

With the mixture of oligosaccharides from RNAase B, owing to a lack of solubility in the anhydrous condition, the inclusion of water (5%, v/v) and an elevated incubation temperature (60 °C) were found necessary for conjugation. Approx. 80% conjugation was achieved under these conditions, and the resulting four orcinol-positive neoglycolipid bands, M5, M6, M7 and M8, were

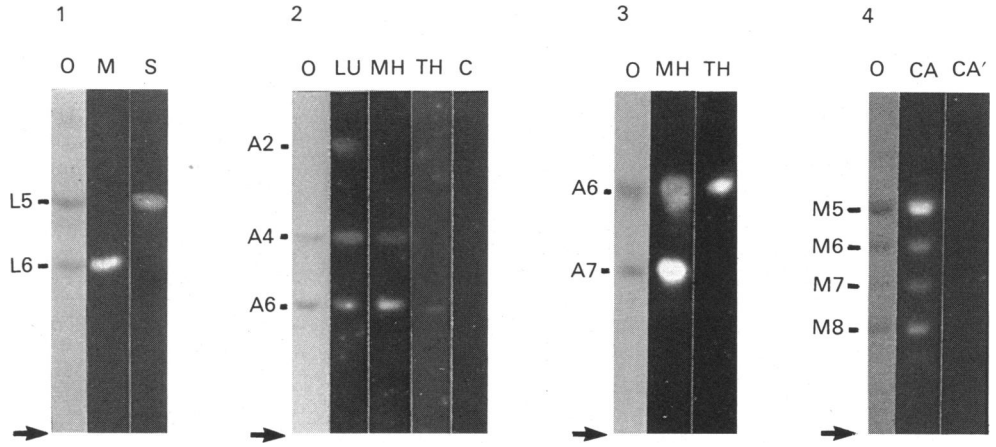

Fig. 3. T.l.c. showing specificity of reactions of neoglycolipids with monoclonal antibodies and the lectin concanavalin A

Mixtures of neoglycolipids were chromatographed in solvent 1 (panels 1 and 2) or solvent 2 (panels 3 and 4). In panels 1–3 chromatogram strips were overlaid with antibodies: anti-MAM-3b (M), anti-SSEA-1 (S), LU-35 (LU), MH-1 (MH), TH-1 (TH) or tissue-culture medium with fetal-calf serum (C) as a negative control, followed by ^{125}I-labelled anti-mouse antibodies. In panel 4 the chromatogram strips were overlaid with ^{125}I-labelled concanavalin A in the absence (CA) or the presence (CA′) of 200 mM-α-methyl mannoside. Reactivities of ^{125}I-labelled antibodies or lectin were detected by autoradiography. Selected strips were later stained with orcinol (as shown in lanes O); these were: a separate strip, panel 1; strip LU, panel 2; strip MH, panel 3; strip CA, panel 4. The amount of each neoglycolipid applied per lane (measured as carbohydrate) was 1 μg, except for lane M (0.1 μg), lane S (0.03 μg) and panel 4 (2.5 μg). Autoradiography was for 16 h (panels 1 and 4), 48 h (panel 2 and MH in panel 3) and 108 h (TH in panel 3). Abbreviations: A2, A4, A6 and A7, blood-group A di-, tetra-, hexa- and hepta-saccharides respectively; L5 and L6, lacto-*N*-fucopentaose III and lacto-*N*-difucohexaose I respectively; M5, M6, M7 and M8, high-mannose type oligosaccharides with five, six, seven and eight mannose residues respectively.

clearly reactive with ^{125}I-labelled concanavalin A (Fig. 3, panel 4). The specificity of this reaction was confirmed by (*a*) abolition of the reaction in the presence of 200 mM-α-methyl mannoside and (*b*) identification of the four components as the PPEADP conjugates of $Man_5GlcNAc_2$, $Man_6GlcNAc_2$ $Man_7GlcNAc_2$ and $Man_8GlcNAc_2$ by fast-atom-bombardment mass spectrometry performed directly on the silica plates (A. M. Lawson, T. Mizuochi & T. Feizi, unpublished work), in accordance with previous knowledge of the *N*-linked oligosaccharide structures of this glycoprotein (Liang *et al.*, 1980). These oligosaccharides respectively contain three, four, five and six α-mannosyl residues non-substituted at their C-3, C-4 and C-6 positions. The presence of at least two such residues is required for binding to concanavalin A (Ogata *et al.*, 1975).

DISCUSSION

Since conjugation by reductive amination involves water elimination:

$$R_1\text{-}NH_2 + CHO\text{-}R_2 \xrightarrow{NaCNBH_3} R_1\text{-}NH\text{-}CH_2\text{-}R_2 + H_2O$$

anhydrous conditions would be expected to enhance the reaction rate. Borch *et al.* (1971) made use of this principle by including water-absorbing molecular sieves to accelerate conjugation reactions between highly hindered cyclic ketones, such as norbornanone, and ammonia or dimethylamine. Wiegandt & Ziegler (1974) reported that successful conjugation of sialyl-lactose to stearylamine (in 50% yield) was achieved by refluxing in methanol for 2 h. For conjugating dextran oligomers to stearylamine in water-containing reaction mixtures [29% (v/v) water in tetrahydrofuran] an incubation time of 3 weeks was required (Wood & Kabat, 1981).

Our studies clearly show that effective conjugation of di- to hepta-saccharides to PPEADP can be achieved in 16 h by using an organic solvent [chloroform/methanol (1:1, v/v)] in the absence of water. A possible additional factor in the enhanced conjugation rate with PPEADP by using the anhydrous organic condition is that the reductive amination of aliphatic amines is favoured, as observed by Kallin *et al.* (1986). With larger oligosaccharides, however, a lack of solubility in chloroform/methanol means that the inclusion of some water or the use of alternative solvent systems and reaction conditions is required. Thus the hepta- to deca-saccharide mixture obtained from RNAase B could be successfully conjugated in the presence of water (5%, v/v) and by raising the incubation temperature to 60 °C. Traces of monoacylation products derived from PPEADP and from the neoglycolipids are formed under both conjugation conditions (anhydrous and hydrous) described in the present report. These migrate with R_F values about half of those in the parent compounds. They are detected in heavily loaded thin-layer chromatograms and have been identified by fast-atom-bombardment mass spectrometry (A. M. Lawson, T. Mizuochi & T. Feizi, unpublished work). However, their presence at such low concentrations did not give rise to additional binding reactivities in these experiments.

The present study, using well-characterized monoclonal antibodies and chromatographic systems that separate free oligosaccharides and free lipid from neoglycolipids, has shown the efficiency of oligosaccharide conjugation as well as the sensitivity and specificity of antigen detection with the neoglycolipids. In addition, by using antibody TH-1, immunochemical evidence has been obtained for the occurrence of an oligosaccharide with the repetitive blood-group A sequence in contents of the human fetal gastrointestinal tract, from where the blood-group A hexasaccharide had been isolated. This sequence has thus far been reported (Clausen *et al.*, 1985) only among oligosaccharides of human erythrocyte membranes. The ability to construct efficiently probes from *N*-linked oligosaccharides released from glycoprotein opens the way to studies of their recognition by diverse carbohydrate-binding proteins of animal, plant and microbial origins.

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