Oligolysine-based saccharide clusters: synthesis and specificity

Natacha FRISON¹, Philippe MARCEAU, Annie-Claude ROCHE, Michel MONSIGNY and Roger MAYER

Glycobiologie, Vectorologie et Trafic intracellulaire, Centre de Biophysique Moléculaire, CNRS, 1, rue Charles Sadron, F-45071 Orléans Cedex 2, France

In search of specific and highly selective sugar clusters for cell receptors, such as membrane lectins, various disaccharides were coupled to small peptide cores through an amide bond. In a first step, the reducing disaccharides, i.e. lactose and three different dimannoses, were converted into glycosyl-pyroglytamyl- β -alanine derivatives. The free carboxylic group of these conjugates was then coupled to the α and ϵ amino groups of the core peptide (Lys_n-Ala-Cys-NH₂) with $n = 1$ to 5, with complete substitution leading to homogeneous glycoclusters. The thiol group of the cysteine residue was used to tag the glycosylated oligolysines upon reaction with fluorescein iodoacetamide. The affinity of these glycoclusters towards two plant lectins was assessed by

surface plasmon resonance. The selectivity of their cell uptake was investigated by flow cytometry using two types of cells: a human hepatoma cell line (HepG2 cells) expressing the plasma membrane galactose-specific lectin, and monocyte-derived dendritic cells expressing the plasma membrane mannose-specific lectin. The glycoclusters containing four or five disaccharides were shown to bind plant lectins and cell surface membrane lectins with a narrow selectivity and with a high affinity.

Key words: endocytosis, glycosynthons, flow cytometry, lectins, surface plasmon resonance.

INTRODUCTION

High affinity ligands for cell specific receptors, such as membrane lectins, belong amongst either complex oligosaccharides of natural origin, or synthetic clusters made of sugar units attached either to a protein (neoglycoprotein) or to a small peptide core. Knowing that sugar–lectin interactions depend on the density of the partners, several teams have synthesized multivalent glycopeptides by coupling, for instance, monosaccharide derivatives on to oligolysines [1–3]. Small multivalent synthetic glycopeptides with two to four mannose residues covalently linked through a spacer arm to the α - and ϵ -amino groups of lysine, di-, and tri-lysine, were shown to be competitive inhibitors of rat alveolar macrophage uptake of a neoglycoprotein (mannosylated BSA) with 50% inhibition in the micromolar concentration range, while the galactosyl-peptide analogue did not inhibit the uptake of mannosylated BSA [4], in agreement with the fact that macrophages did not recognize galactose residues.

In search of synthetic high affinity ligands for a human mannose receptor isolated from placenta, Biessen and co-workers [5] synthesized a series of homologous lysine-based monosaccharides containing two to six terminal α -D-mannose groups linked to the α - and ϵ -amino groups of a linear oligolysine through a phenylthiocarbamyl arm. The synthesized α -mannose clusters were able to displace the binding of ribonuclease B and tissue-type plasminogen activator from the isolated human mannose receptor.

In order to improve both the specificity and the selectivity of sugar clusters, we decided to use disaccharides instead of monosaccharides. With the aim of avoiding the use of a phenyl-

thiocarbamyl bond [5] which may lead to cytotoxic derivatives, we synthesized *N*-glycosyl-pyroglytamyl derivatives [6] bearing a C-terminal carboxylic group [7] able to be coupled to an amino group. In this approach, it is possible to obtain clusters in which the core ends with one reacting group which allows the cluster to be linked on to a molecule, such as a gene vector, an oligonucleotide, or a matrix. In a previous study [7], we showed that both natural oligomannosides and oligolysine partially substituted with a dimannose derivative were taken up by dendritic cells.

In this paper, we describe the synthesis of diglycosylpyroglytamyl- β -alanine derivatives (glycosynthons) and an improved synthesis of glycoclusters by coupling glycosynthons either to lysine or to an oligolysine containing up to five lysyl residues (Lys_n-Ala-Cys-NH₂ peptide), leading to fully substituted biodegradable conjugates. In order to test the specificity and the selectivity, we synthesized four types of clusters: one containing lactose and three containing α 2, α 3 or α 6 dimannose. With the aim of designing a high affinity cluster at the lowest possible cost, we synthesized a series of clusters containing two to six sugar moieties. The peptide core ended with a cysteine residue, the thiol group of which was either substituted with a pyridylthio group or with an acetamidofluorescein group.

Two main properties of the clusters were investigated: (i) their affinity towards two immobilized plant lectins, a galactose-specific lectin *Ricinus communis* agglutinin (Rca) and a mannose-specific lectin *Canavalia ensiformis* agglutinin (ConA), assessed by surface plasmon resonance (SPR); and (ii) their selective uptake by HepG2 cells (a human hepatoma cell line) expressing a cell surface galactose-specific lectin [8] and blood

Abbreviations used: Boc, t-butoxycarbonyl; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; ConA, concanavalin A; DC-SIGN, dendritic cell-specific ICAM-grabbing non-integrin; DIEA, *N,N*-di-isopropylethylamine; DMEM, Dulbecco's minimum essential medium; DMF, dimethylformamide; EDT, ethanedithiol; ESI-MS, electrospray ionization MS; FCS, fetal calf serum; Glp, pyroglytamate; H- β Ala-Obzl, H- β Ala-benzyl ester; H- β Ala-Obzl, Tos, H- β Ala-Obzl tosylate; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HPAEC, high-performance anion-exchange chromatography; Fmoc, fluorenyl-9-methoxycarbonyl; IAF, 5-(iodoacetamido) fluorescein; Lac, lactose; NMPO, *N*-methylpyrrolidone; Os, saccharide; OtB, *t*-butyl; PBMC, peripheral blood mononuclear cells; Rca, *Ricinus communis* agglutinin; RU, resonance units; SPR, surface plasmon resonance; SPy, 2-thiopyridyl; TBME, *t*-butyl methyl ether; TCEP, tris-(2-carboxyethyl)phosphine; TFA, trifluoroacetic acid.

¹ To whom correspondence should be addressed (e-mail frison@cnsr-orleans.fr).

monocyte-derived dendritic cells expressing cell surface mannose-specific lectins [9,10], assessed by flow cytometry.

EXPERIMENTAL PROCEDURES

Materials

Lactose and *N,N*-di-isopropylethylamine (DIEA) were purchased from Janssen Chimica (Beerse, Belgium); dimannoses (Man α 2Man, Man α 3Man, Man α 6Man) (Figure 1) were from Dextra Laboratories (Reading, U.K.). Imidazole, DMSO, *n*-butanol, acetic acid, trifluoroacetic acid, ethanedithiol, molecular sieves (4 Å; 1 Å = 0.1 nm) and silica gel 60 were from Merck (Darmstadt, Germany). 2',2'-Dithiodipyridine and monensin were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). *t*-Butyl methyl ether (TBME) was from Carlo Erba (Val de Reuil, France). Acetonitrile was purchased from SDS (Peypin, France). Tris-(2-carboxyethyl)phosphine (TCEP) and 5-(iodoacetamido)fluorescein (IAF) were from Molecular Probes (Leiden, The Netherlands). *N*-Methylpyrrolidone (NMPO) was from Applied Biosystems (Foster City, CA, U.S.A.). Benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were from Richelieu Biotechnologies (Saint-Hyacinthe, QC, Canada). 1-Hydroxybenzotriazole (HOBT) was purchased from Senn Chemicals AG (Diesdorf, Switzerland). Glu- β Ala-benzyl ester (Glu- β Ala-Obzl) was synthesized by classical peptide synthesis in solution, starting with Boc-Glu(OtBu)-OH (where Boc is *t*-butoxycarbonyl and OtB is *t*-butyl) and H- β Ala-Obzl tosylate (H- β Ala-Obzl, Tos) from Bachem (Budendorf, Switzerland). Cell culture medium: Dulbecco's minimum essential medium (DMEM) and fetal calf serum (FCS) were from GIBCO BRL (Grand Island, NY, U.S.A.). Paraformaldehyde, Rca and saponin were from Sigma. Texas Red goat anti-(mouse IgG) and Texas Red goat anti-(rabbit IgG) were from Bio-Rad. Neoglycoproteins (Lac-BSA, Man-BSA) were prepared by coupling BSA with Gal β 4Glc β phenylisothiocyanate and Man α phenylisothiocyanate respectively, and fluorescein-labelled neoglycoproteins (F-Lac-BSA, F-Man-BSA) by substituting Lac-BSA

and Man-BSA with fluorescein isothiocyanate, as previously described [11]. The number of sugar moieties bound per BSA was 25 ± 3 , as determined by the resorcinol sulphuric acid micromethod [12].

Cells

HepG2 human hepatoma cells were obtained from American Type Culture Collection (8055 HB, Rockville, MD, U.S.A.). HepG2 cells were cultured in complete DMEM medium complemented with 5% (v/v) heat-inactivated FCS containing 2 mM L-glutamine and 1% antibiotics (50 units/ml penicillin, 50 μ g/ml streptomycin). Dendritic cells named dendritophages D Φ (IDM, Paris, France) were obtained by apheresis from healthy volunteer peripheral blood mononuclear cells (PBMC) as follows: 1×10^{10} PBMC were washed 3 times in PBS and cultured for 7 days in non-adherent hydrophobic bags (Stedim, Aubagne, France) in serum-free AIM V medium (VacCell medium, IDM, Paris, France) supplemented with 500 units/ml granulocyte macrophage cell stimulating factor ('GM-CSF') and 50 ng/ml interleukin-13, at a density of 5×10^6 cells/ml. Interleukin-13 was added on day 4 of culture. On day 7, D Φ were isolated by elutriation. The purity of D Φ ranged from 75 to 95% and the cell viability was higher than 95%.

Chromatography

Conjugates were purified by gel filtration on either a Trisacryl GF05 column (2 cm $\times$ 45 cm; Biosepra, Villeneuve-la-Garenne, France) stabilized and eluted with distilled water containing 5% *n*-butanol, or a BioGel column (P2, P4 or P6; Bio-Rad, Oxfordshire, U.K.) stabilized and eluted with 50 mM acetic acid. A flow rate of 0.35 ml/min was used.

High-performance anion-exchange chromatography (HPAEC) was performed on a Dionex DX-300 chromatography system (Sunnyvale, CA, U.S.A.) which includes a quaternary gradient pump, an eluent degas (He) module, a CarboPac PA1 column (4 mm $\times$ 250 mm) with a matching guard column, and a pulsed amperometric detector. A flow rate of 1 ml/min was used. The column was equilibrated with solvent A (0.1 M sodium hydroxide) and eluted as follows: solvent A for 5 min, followed by a linear gradient of 0–20% solvent B (1 M sodium acetate in 0.1 M sodium hydroxide) for 10 min, then a linear gradient of 20–100% solvent B for 15 min.

HPLC was performed on a Waters 2690 (Milford, MA, U.S.A.) system linked to a Waters 996 photodiode array detector. An Alltech 5 μ m C₁₈ column (4.6 mm $\times$ 150 mm) (Alltech, Deerfield, IL, U.S.A.) and a Vydac 5 μ m C₄ column (4.6 mm $\times$ 250 mm; Hesperia, CA, U.S.A.) were stabilized at 35 °C in the starting eluent of 2% solvent B [95% acetonitrile/5% water/0.1% trifluoroacetic acid (TFA)] in solvent A (95% water/5% acetonitrile/0.1% TFA). Upon injection, the compounds were eluted by using this mixture for 5 min and then a linear gradient of 2–15% solvent B in solvent A over 15 min (see Table 1). A flow rate of 1 ml/min was used.

Electrospray ionization mass spectrometry (ESI-MS)

The positive ion electrospray mass spectra were obtained using a platform quadrupole mass spectrometer (Quattro II, Micromass, Manchester, U.K.) equipped with a nebulizer-assisted electrospray source. A voltage difference of 3.11 kV was applied between the capillary and the counter electrode. The sample cone voltage was 20–25 V. The ion source was kept at 80 °C. Instrument control and data analysis were accomplished using the Masslynx application software (version 3.4; Micromass). Calibration of

Figure 1 Structures of disaccharides used for synthesis of oligolysine-based saccharide clusters

Table 1 Characteristics of the lysine-based oligosaccharide clusters

* The sugar linked to the Glp nitrogen is a β anomer. † Masses in italics were measured by MALDI-TOF; t_R is retention time. ‡ HPLC elution was performed using a C4 column with a gradient of 2% solvent B (95% CH₃CN/5% H₂O/0.1% TFA) in solvent A (95% H₂O/5% CH₃CN/0.1% TFA) for 5 min, followed by a linear gradient of 2–15% solvent B in solvent A over 15 min; elution was monitored by UV absorbance at 220 nm, 280 nm or 450 nm according to the cysteine side-chain substituent, using a photodiode array detector. Values in parentheses are the masses of minor components. Spy, 2-thiopyridyl; Flu, fluoresceinyl.

Oligosyl clusters*			Molecular mass		t_R ‡ (min)
			Calculated	Measured†	
Lac-Glp- β Ala-[Lys(Lac-Glp- β Ala)]AlaCys(R)-NH ₂	R = -Spy	5'a	1440.5	1440.6	8.3
	R = -Flu	5a	1718.6	1719.2	20.7
Lac-Glp- β Ala-[Lys(Lac-Glp- β Ala)] ₂ AlaCys(R)-NH ₂	R = -Spy	5b	2074.8	2075.8	9.8
	R = -Flu	5'b	2352.9	2353.9	20.0
Lac-Glp- β Ala-[Lys(Lac-Glp- β Ala)] ₃ AlaCys(R)-NH ₂	R = -Spy	5c	2709.1	2708.8	12.3
	R = -Flu	5'c	2987.1	2989.3	19.4
Lac-Glp- β Ala-[Lys(Lac-Glp- β Ala)] ₄ AlaCys(R)-NH ₂	R = -Spy	5d	3343.3	3342.9 (+ 2878.4)	12.8 (+ 14.3)
	R = -Flu	5'd	3621.4	3622.8	19.1
Lac-Glp- β Ala-[Lys(Lac-Glp- β Ala)] ₅ AlaCys(R)-NH ₂	R = -Spy	5e	3977.6	3978.3 (+ 3513.7)	13.5 (+ 15.0)
	R = -Flu	5'e	4255.7	4256.6 (+ 3792.7)	19.1
Lac-Glp- β Ala-[Lys(Lac-Glp- β Ala)] ₅ Cys(R)-NH ₂	R = -Spy	5f	4261.9	4261.1 (+ 3754.0)	19.1 (+ 19.7)
	R = -Flu	5'f	4540.0	4546.0 (+ 4035.5)	23.4 (+ 24.6)
Man α 6Man-Glp- β Ala-[Lys(Man α 6Man-Glp- β Ala)] ₄ AlaCys(R)-NH ₂	R = -Spy	6d	3343.3	3346.5	10.6
	R = -Flu	6'd	3621.4	3622.7 (+ 3158.4)	17.6
Man α 3Man-Glp- β Ala-[Lys(Man α 3Man-Glp- β Ala)] ₄ AlaCys(R)-NH ₂	R = -Spy	7d	3343.3	3345.4	11.8
	R = -Flu	7'd	3621.4	3623.2	19.4
Man α 2Man-Glp- β Ala-[Lys(Man α 2Man-Glp- β Ala)] ₄ AlaCys(R)-NH ₂	R = -Spy	8d	3343.3	3346.4	11.0
	R = -Flu	8'd	3621.4	3622.3	18.1

the mass spectrometer was performed using horse myoglobin (Sigma). Samples were dissolved in a 4:1 (v/v) acetonitrile/water mixture containing 0.1% (v/v) formic acid. ESI mass spectra were run in both positive and negative ion modes. Clusters were also characterized by matrix-assisted laser-desorption/ionization time-of-flight ('MALDI-TOF') with a Biflex III spectrometer (Bruker, Wissembourg, France), in positive mode, using α -cyano-4-hydroxycinnamic acid as a matrix.

¹H NMR spectroscopy

For ¹H NMR analysis, compounds were dissolved at 25 °C in deuterated water, once with D₂O containing 99.9% D and, after freeze-drying, with D₂O containing 99.96% D (Sigma). ¹H NMR spectroscopy was performed at 300 K with a Varian Unity 500 MHz NMR spectrometer.

TLC

TLC was performed using silica gel F₂₅₄ preformed layers on a plastic sheet (Merck, Darmstadt, Germany), and the chloroform/methanol/water (13:8:2, by vol.) mixture was used as an eluent. Carbohydrates were visualized upon spraying with ammonium heptamolybdate/H₂SO₄ and subsequent heating. Compounds containing free amino groups were visualized by ninhydrin spray and subsequent heating at 80 °C.

Flow cytometry

Adherent cells (4 × 10⁵ HepG2 on day 1 after plating, or DΦ on day 3 after elutriation) were incubated for 2 h at 37 °C in complete culture medium in the presence of the fluorescein-labelled conjugates (0.05–20 µg/ml). After washing in PBS, the cell-associated fluorescence was determined by flow cytometry after a final incubation for 30 min at 4 °C in the presence or absence of 50 µM monensin [13,14]. The cell-associated fluorescence was analysed with a Becton Dickinson-LSR flow cyto-

meter and data were analysed using the Cell Quest Software (Becton Dickinson, Le-Pont-De-Claix, France).

SPR

BIACore 2000 (BIAcore, Uppsala, Sweden) was used to quantify interactions between lysine-based oligosaccharide clusters and lectins. Lectins were immobilized at pH 4.5 (10 mM sodium acetate buffer) on a CM5 sensor chip (BIAcore). The chip carboxymethylated dextran matrix was activated and derivatized by using the amine coupling method [mixture of 100 mM *N*-hydroxysuccinimide (Sigma-Aldrich) and 400 mM *N*-ethyl-*N'*-(dimethylaminopropyl)-carbodi-imide (Acros, Noisy-le-Grand, France)]; the remaining activated sites were then blocked with 1 M ethanolamine, pH 8.5 (Sigma-Aldrich). The binding of any material on the chip induced a refraction index increase, expressed as resonance units (RU). All measurements, using the sensor surfaces, were carried out in 10 mM Hepes Balanced Saline buffer [10 mM Hepes (pH 7.4), 1 mM CaCl₂, 1 mM MgCl₂, 150 mM NaCl, 17 mM Na₂SO₄, 0.5% (v/v) surfactant P20 (BIAcore)]. Analysis was performed at 25.0 °C and at a flow rate of 20 µl/min. In the BIAcore instrument, the sensor chip is divided into four flow cells which can be used to individually measure interactions. Flow cell number one was used as a control flow cell and treated in an analogous manner except that the lectin addition step was omitted. Analyte samples were prepared and diluted in the running buffer, pH 7.4. Surfaces were regenerated with 30 µl of 0.3 M lactose for Rca and 0.3 M α -methylmannoside (Sigma) for ConA. Glycoclusters which have a low molecular mass do not give a significant signal. Also, we favoured a procedure based on the inhibition of the binding of a neoglycoprotein on an immobilized lectin. Inhibition constants were derived from experiments using analytes in a 0.25–100 µM concentration range, using a serial 3-fold dilution in running buffer containing 10 µg/ml (approx. 0.125 µM) neoglycoprotein Lac-BSA or Man-BSA, for Rca and ConA respectively; this

concentration is the half-saturation concentration which gives the half-maximal value, $RU_{\max}/2$.

In the case of a classical association, the Langmuir equation [15] can be written, according to Scatchard [16], as

$$K \cdot (n-r) = r \cdot g^{-1} \text{ or } r \cdot (n-r)^{-1} = K \cdot g \quad (1)$$

where K is the affinity constant between a lectin and a neoglycoprotein expressed as $\text{litre} \cdot \text{mol}^{-1}$, n is the total number of sites on the sensor chip, r is the number of sites occupied by a neoglycoprotein and g the concentration of the neoglycoprotein used as an analyte, expressed as $\text{mol} \cdot \text{litre}^{-1}$.

It is well known from the law of mass action that a linear relationship between free (f) and bound (b) hapten concentrations allows the determination of the equilibrium constant K when the combination of a hapten and an antibody is the same for all the combining sites, i.e. that there is no heterogeneity. More often, the plot $1/b$ versus $1/f$ exhibits a downward curvature attributed to combining sites heterogeneity [17]. The same behaviour is relevant in the case of neoglycoproteins and lectins. In this case, the heterogeneity comes from the neoglycoprotein which contains 25 sugars on average, and from a non-homogeneous density of immobilized lectins on the chip. The number of sugars present in a neoglycoprotein varies from one molecule to another, because about 25 lysines ± 3 are glycosylated and more than 25 are not. Therefore the heterogeneity comes from a quantitative aspect, but also from a qualitative one: the position of the sugar moieties on the neoglycoprotein. Moreover, the interaction between such a neoglycoprotein and a lectin is complex because of an avidity effect [18,19].

Nisonoff and Pressman [17] used the distribution function of Sips [20], in which the data may be fitted to a straight line. The equation proposed by Sips is derived from the adsorption of a gas on a solid surface, but it may be transformed into an equation applicable to equilibria in solution, as is the case in SPR experiments where RU is measured at equilibrium.

According to eqn. 1,

$$\log[r \cdot (n-r)^{-1}] = \alpha \cdot \log(K \cdot g) \quad (2)$$

or

$$r \cdot (n-r)^{-1} = (K \cdot g)^{\alpha} \quad (3)$$

Since $RU_{\max}$ is related to n and RU is related to r , it follows that $RU_{\max} \cdot (RU)^{-1} = n \cdot r^{-1}$, so eqn. 3 gives

$$[RU_{\max} \cdot (RU)^{-1} - 1]^{1/\alpha} = (K \cdot g)^{-1} \quad (4)$$

The slope α is called either the heterogeneity coefficient or the Sips coefficient.

For a homogeneous group of sites, α is equal to +1; decreasing values of α correspond to increasing degrees of heterogeneity, showing an avidity effect or reflecting a heterogeneity either related to the ligands or to the receptors. The lowest value of α corresponds to the highest heterogeneity.

The association constant of the inhibitor K_i was calculated from the apparent affinity K of a neoglycoprotein relevant for the immobilized lectin, measured in the presence of glycosylated clusters. In the presence of an inhibitor i , with an association constant K_i , the saturation equations giving K and K_i , become

$$K \cdot (n-r-q) = r \cdot g^{-1} \quad (5)$$

and

$$K_i \cdot (n-r-q) = q \cdot i^{-1} \quad (6)$$

where q is the number of sites occupied by a glyocluster and is equal to

$$q = r \cdot i \cdot K_i \cdot (K \cdot g)^{-1} \quad (7)$$

and

$$K \cdot [n-r-r \cdot i \cdot K_i \cdot (K \cdot g)^{-1}] = r \cdot g^{-1} \quad (8)$$

therefore

$$(n-r) \cdot r^{-1} = (K \cdot g)^{-1} + i \cdot K_i \cdot (K \cdot g)^{-1} \quad (9)$$

When the binding is heterogeneous, eqn. 9 becomes

$$[(n-r) \cdot r^{-1}]^{1/\alpha} = (1 + i \cdot K_i) \cdot (K \cdot g)^{-1} \quad (10)$$

K_i is deduced from

$$(RU_{\max} \cdot RU^{-1} - 1)^{1/\alpha} = (1 + i \cdot K_i) \cdot (K \cdot g)^{-1} \quad (11)$$

Synthesis, purification and characterization of peptide cores

A series of peptide cores (Lys) $_n$ -Ala-Cys with $n = 1$ to 5, and (Lys-Ala) $_5$ -Cys with a C-terminal amide, were prepared by solid phase synthesis on an Applied 433-A peptide synthesizer (Foster City, CA, U.S.A.) equipped with a conductimetric monitor, using a fluorenyl-9-methoxycarbonyl (Fmoc) strategy, and starting from an Fmoc-amide resin (0.64 mmol/g) (Applied Biosystems). Fmoc-protected amino acids [Lys(Boc) and Cys(trityl)] were from Senn Chemicals (Dielsdorf, Switzerland). A cysteine residue was appended at the C-terminal end of the peptide to enable labelling of the conjugate with a fluorescent tag.

The peptide was deprotected and released from the resin by adding 5 ml of a TFA/ethanedithiol (EDT)/water mixture (190/5/5) for 3 h at 25 °C under stirring. The solid support was filtered off and washed with a TFA/EDT/water mixture. Peptides were precipitated from the filtrate by adding TBME. The precipitate was collected by centrifugation and washed thoroughly with TBME. The cysteine side chain was further protected with a 2-thiopyridyl (-SPy) group which will be easily reduced with TCEP. The unprotected peptide was dissolved in water/methanol (1:1, v/v) under nitrogen. A degassed solution of 2,2'-dithiodipyridine (10 equivalents) in water/methanol (4:1, v/v) was added and the pH of the reaction mixture was increased to 8.2 with diluted NH_4OH . The mixture was stirred under nitrogen for 3 h. The excess of dithiodipyridine and the pyridine-2-thione formed were extracted with ethyl acetate, whereas the aqueous phase containing the protected peptide was concentrated and freeze-dried. Peptides were analysed by HPLC and MS.

Synthesis of glutamyl- β -alanyl-benzyl ester (H-Glu- β Ala-OBzl) (1)

The title compound was obtained by coupling Boc-Glu(OtBu)-OH with H- β Ala-OBzl.Tos. Briefly, Boc-Glu(OtBu)-OH (3.03 g, 10 mmol) was dissolved in 20 ml of ethyl acetate and coupled with H- β Ala-OBzl.Tos (3.51 g, 10 mmol) in the presence of N,N -dicyclohexylcarbodi-imide (2.2 g, 11 mmol) and 1.74 ml (10 mmol) of DIEA. After stirring overnight, the coupling efficiency was assessed by TLC in chloroform/methanol (9:1, v/v). The R_f of the dipeptide was 0.86 and the reaction was completed within 18 h. The insoluble by-products were eliminated by filtration. The filtrate was washed twice with 40 mM KHSO_4 (25 ml), twice with distilled water (25 ml), twice with 60 mM NaHCO_3 (25 ml) and twice again with distilled water (25 ml). The organic layer was dried on sodium sulphate, filtered and evaporated under reduced pressure. The dipeptide Boc-Glu(OtBu)- β Ala-OBzl was then dissolved in 30 ml of water/trifluoroacetic acid (5:95, v/v) and kept for 1 h at 25 °C leading to H-Glu- β Ala-OBzl, TFA. The progress of the reaction was assessed by TLC in chloroform/methanol/water (13:8:2, by vol.): finally, a single spot was seen both under UV and upon staining with ninhydrin ($R_f = 0.56$). The solvent was evaporated under reduced pressure, the solid material was dissolved in 3 ml

Figure 2 Synthesis of lysine-based oligosaccharide clusters

$\text{R}_1 = \text{-H, -OH or Man}\alpha\text{-D-}; \text{R}_2 = \text{-H, Man}\alpha\text{- or Gal}\beta\text{-}. \text{Os} = \text{lactosyl (5 and 5'), dimannosyl Man}\alpha\text{6Man (6 and 6'), Man}\alpha\text{3Man (7 and 7'), or Man}\alpha\text{2Man (8 and 8')}.$

of chloroform and precipitated at -20°C upon adding TBME. The expected product was obtained in 90% yield.

Synthesis of oligosaccharyl-pyroglutamate (Glp)- β Ala-OBzl (2) and (3)

In a typical experiment (see Figure 2) according to Quétard et al. [21], the disaccharide (50 mg, 146 μmol), H-Glu- β Ala-OBzl (90 mg, 292 μmol) and imidazole (40 mg, 584 μmol) were dissolved in 400 μl of NMPO. The solution was kept at 50°C for 6 h. Without any purification, the glycosylamine formed (2) was then intramolecularly *N*-acylated by the γ -carboxyl group of glutamic acid, at 25°C , by adding 146 mg (330 μmol) of BOP and 23 mg (330 μmol) of imidazole. The reaction was monitored by HPAEC and was complete within 1 h. The glycoconjugate was precipitated from its NMPO solution by pouring it in 10 vol. of TBME; under such conditions, imidazole and hydroxybenzotriazole remained in the supernatant. Disaccharyl-Glp- β Ala-OBzl (3) was purified by gel filtration on a Trisacryl GF 05 column (2 cm $\times$ 45 cm; Biosepra, Villeneuve-la-Garenne, France), stabi-

lized and eluted with distilled water containing 5% *n*-butanol. The yield was 80–90%, depending on the disaccharide used. By using ESI-MS, for the lactosyl-Glp- β Ala-OBzl, the observed mass of the molecular ion was 615.3 (615.2 calculated for $\text{C}_{27}\text{H}_{38}\text{N}_2\text{O}_{14}$ [$M + \text{H}^+$]). ^1H NMR (500 MHz, $^2\text{H}_2\text{O}$), δ (p.p.m.): 7.44 (5 H, s, *H* aromatic), 5.18 (2 H, s, $-\text{OCH}_2$ Bzl), 5.10 (1 H, d, H_1 - β -D-Glc), 4.65 (1 H, dd, $J_{1,2} = 7.8$ Hz, αCH Glp), 2.83 (1 H, m, γCH_2 Glp), 2.56 (2 H, m, $\gamma'\text{CH}_2$ Glp and βCH_2 Glp), 2.28 (1 H, m, $\beta'\text{CH}_2$ Glp).

Reduction of oligosaccharyl-Glp- β Ala-OBzl (4)

Compound (3) was dissolved in 10 ml of a water/methanol mixture (1:1, v/v). The solution was purged under nitrogen for 30 min. Then, 25 mg (10%) of palladium/charcoal (Merck) was added to this solution. The suspension was stirred for 3 h under hydrogen at 25°C . The reaction was monitored by TLC in chloroform/methanol/water (13:8:2, by vol.). The glycosynthion had an R_F of 0.30. The palladium/charcoal mixture was removed by filtration, the methanol was removed under reduced pressure and the methanol-free solution containing the product (4) was freeze-dried. The yield was 95%. The mass of the molecular ion determined by ESI-MS [calculated for $\text{C}_{20}\text{H}_{32}\text{N}_2\text{O}_{14}$ ($M + \text{H}^+$), 525.2] was 525.3; the deprotection of the C-terminal carboxylic group was shown to be complete by ^1H NMR (500 MHz, $^2\text{H}_2\text{O}$).

Coupling of the oligosaccharyl-Glp- β Ala-OH on to an oligolysine leading to the cluster (5)

The synthesis of these oligosaccharide clusters is shown in Figure 2. The oligolysines used were $\text{Lys}_n\text{-Ala-Cys(Spy)-NH}_2$ with $n = 1$ to 5 and $(\text{Lys-Ala})_5\text{-Cys(Spy)-NH}_2$. A solution of oligolysine (2 μmol) in NMPO (200 μl) with one equivalent of DIEA per amino group was added to disaccharyl-Glp- β Ala-OH (1.5 equivalents per $-\text{NH}_2$) dissolved in NMPO (200 μl) in the presence of 1.5 equivalent of DIEA, 1.5 equivalent of HOBT/HBTU, and 70 mg of 4 Å molecular sieves. The mixture was gently stirred at 25°C for 7 h. The reaction was monitored by TLC in chloroform/methanol/water (13:8:2, by vol.). The cluster was then precipitated by pouring the solution into 10 vol. of TBME and recovered upon centrifugation. The product was purified by gel filtration on a BioGel column (2 cm $\times$ 45 cm; Bio-Rad, Oxfordshire, England), stabilized and eluted with 50 mM acetic acid. The product collected was freeze-dried and analysed by reverse-phase HPLC and MS. Table 1 summarizes the physico-chemical characteristics of the synthesized clusters.

Labelling of oligosaccharyl-Glp- β Ala-[Lys(oligosaccharyl-Glp- β Ala)] $_n$ -Ala-Cys(Spy)-NH $_2$

The lysine-based oligoside cluster (0.6 mmol) was dissolved in 400 μl of phosphate buffer (50 mM, pH 7.2), then 50 μl of dimethylformamide (DMF) was added. The solution was gassed with nitrogen for 30 min; meanwhile a solution of TCEP (1.2 mmol, 0.34 mg) in 100 μl of phosphate buffer (50 mM, pH 7.2) was also gassed with nitrogen. This solution was added to the lysine-based oligoside cluster solution in order to reduce the disulphide bridge. The mixture was stirred under nitrogen for 2 h. A solution of IAF (3 mmol, 1.5 mg) in 200 μl of DMF, gassed with nitrogen, was added on the reduced lysine-based oligoside cluster. The mixture was stirred at 25°C overnight. The crude product was purified on a BioGel P2 column (2 cm $\times$ 45 cm), stabilized and eluted with acetic acid (50 mM). The collected product was finally freeze-dried. The same procedure was used for labelling the cluster Lac β -Glp- β Ala-[Lys(Lac β -Glp- β Ala)-Ala] $_5$ -Cys(Spy)-NH $_2$. The characteristics of the syn-

thesized fluoresceinylated clusters are reported in Table 1. The concentration of fluorescein-labelled clusters was determined from their UV absorbance at 490 nm at pH 7.4 (fluorescein ϵ_{490} : 80000). The affinity of these compounds for the lectins was analysed by SPR and their cell uptake was investigated by flow cytometry.

RESULTS

Structure of oligolysine-clusters

^1H NMR showed that, in glycosynthons, the sugar linked to Glp is in a β configuration. The yield of glycosynthons was almost quantitative, except for Man α 2Man-Glp- β Ala-OH, for which the yield was 60 %, and both α and β configurations were present. This may be due to the compact shape of Man α 2Man which is less extended than the other disaccharides [22] (see Figure 1). The oligolysine-based saccharide clusters were synthesized starting from a series of peptide cores, Lys $_n$ -Ala-Cys-NH $_2$ with $n = 1$ to 5, and (Lys-Ala) $_3$ -Cys-NH $_2$, with the aim of investigating the influence of the number and spatial distribution of the oligosaccharides on their cell uptake. They were characterized by HPLC, ^1H NMR, ESI-MS and matrix-assisted laser-desorption ionization-time-of-flight ('MALDI-TOF') MS (see Table 1). When the synthesis of oligolysine clusters was conducted without a dehydrating agent, the substitution of the amino groups of oligolysine reached a plateau at about 50 % completion, as determined by ^1H NMR from the ratio of the anomeric proton of the sugar at 5.1 p.p.m. and that of the ϵ -CH $_2$ protons of the lysine residues at 3.1 p.p.m.: 3.5 glycosynthons were bound on a pentalysine peptide, instead of the expected six [7]. When the reaction was conducted in the presence of Na $_2$ SO $_4$ as a dehydrating agent, no reaction occurred, as monitored by TLC. Conversely, in the presence of 4 Å molecular sieves, the synthesis of the lysine-based oligosaccharide clusters led to a full substitution of the peptide core (see Table 1). This can be explained by the dehydrating power of molecular sieves and by their basic properties, allowing H $^+$ /Na $^+$ exchange. Traces of peptide cores substituted with n glycosynthons instead of $(n+1)$ were sometimes present with the tetra- or penta-lysine cores.

SPR

SPR allows the determination of both the time course of the binding and the affinity constants. ConA and Rca were linked to the sensor chip, starting with a lectin concentration of 100 $\mu\text{g}/\text{ml}$ and leading to a large increase in the refractive index: $\Delta\text{RU} = 3500$. The neoglycoprotein in the absence or presence of inhibitors was injected at a flow rate of 20 $\mu\text{l}/\text{min}$ in order to avoid any mass transfer problems. The duration of both the binding and the dissociation steps was set at 360 s to reach a plateau value. The regeneration of the chip was achieved by using 0.3 M lactose and 0.3 M α -methylmannopyranoside for the Rca and ConA flow cells respectively. This large concentration of regeneration agent completely washes out any ligand from the flow cell without the loss of any immobilized lectin, owing to the neutral nature of such agents. The binding specificity of the neoglycoproteins was controlled by passing different concentrations of Man-BSA on immobilized Rca and Lac-BSA on ConA: in both cases, there was no detectable increase in refractive index. The number of resonance units (RU) was obtained from the sensorgrams and RU_{max} deduced from the intercept of the linear transformation: $(1/\text{RU})$ versus $(1/g)^\alpha$. The heterogeneity coefficient was $\alpha = 0.55$ for the Lac-BSA binding on to immobilized Rca and $\alpha = 0.7$ for the Man-BSA binding on to immobilized ConA. The neoglycoprotein concentration (10 $\mu\text{g}/\text{ml}$) corres-

Figure 3 Analysis of Lac-BSA binding on Rca with the glycocluster inhibitor Lac-Glp- β Ala-[Lys(Lac-Glp- β Ala)] $_3$ -Ala-Cys(SP)NH $_2$

Binding analysis of a sensorgram corresponding to the inhibition of the binding of Lac-BSA (10 $\mu\text{g}/\text{ml}$) on Rca with the glycocluster inhibitor Lac-Glp- β Ala-[Lys(Lac-Glp- β Ala)] $_3$ -Ala-Cys(SP)NH $_2$ at concentrations of 0, 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 M. Resp. Diff. stands for response difference.

Table 2 Inhibition of the interaction of immobilized Rca with LacBSA, by lysine-based lactose clusters

The association constants (K_i) of the inhibitors were determined by surface plasmon resonance. The enhancement factors were quite similar for a given glycocluster with a variation within $\pm 10\%$ between independent experiments. The compound in bold is the most effective inhibitor, owing to its high affinity and small size.

Lysine-based lactose clusters	K_i (litre $\cdot$ mol $^{-1}$)
Lactose	1.0×10^3
Lac- β -Glp- β Ala-[Lys(Lac- β -Glp- β Ala)] $_1$ -Ala-Cys(SP)-NH $_2$	4.5×10^5
Lac- β -Glp- β Ala-[Lys(Lac- β -Glp- β Ala)] $_2$ -Ala-Cys(SP)-NH $_2$	4.0×10^5
Lac- β -Glp- β Ala-[Lys(Lac- β -Glp- β Ala)] $_3$ -Ala-Cys(SP)-NH $_2$	1.0×10^6
Lac-β-Glp-βAla-[Lys(Lac-β-Glp-βAla)]$_4$-Ala-Cys(SP)-NH$_2$	3.0×10^6
Lac- β -Glp- β Ala-[Lys(Lac- β -Glp- β Ala)] $_5$ -Ala-Cys(SP)-NH $_2$	3.0×10^6

Table 3 Inhibition of the interaction of immobilized ConA with ManBSA, by lysine-based dimannose clusters

The association constants (K_i) of the inhibitors were determined by surface plasmon resonance. The enhancement factors were quite similar for a given glycocluster with a variation within $\pm 10\%$ between independent experiments.

Lysine-based dimannose clusters	K_i (litre $\cdot$ mol $^{-1}$)
Man α 6Man- β -Glp- β Ala-[Lys(Man α 6Man- β -Glp- β Ala)] $_4$ -Ala-Cys(SP)-NH $_2$	5.5×10^4
Man α 3Man- β -Glp- β Ala-[Lys(Man α 3Man- β -Glp- β Ala)] $_4$ -Ala-Cys(SP)-NH $_2$	5.0×10^4
Man α 2Man- β -Glp- β Ala-[Lys(Man α 2Man- β -Glp- β Ala)] $_4$ -Ala-Cys(SP)-NH $_2$	3.0×10^5

ponding to $\text{RU}_{\text{max}}/2$ was chosen to perform inhibition experiments with glycosylated clusters (Figure 3).

It appears that the lactose-based clusters containing either four or five lysines have the highest affinity towards Rca, which is 2×10^3 times larger than that of free lactose (Table 2). The binding constant of dimannosyl clusters onto ConA was deduced from their inhibitory effect on the binding of mannosylated bovine serum albumin on immobilized ConA. Man α 2Man- β -Glp- β Ala-[Lys(Man α 2Man- β -Glp- β Ala)] $_4$ -Ala-Cys(SP)-NH $_2$

Figure 4 (A) Uptake of oligokislactose clusters by HepG2 cells and (B) uptake of oligokisdimannose clusters by DΦ cells

(A) A representative experiment of the uptake of oligokislactose clusters by HepG2 cells. HepG2 cells (2×10^5) were incubated for 2 h at 37 °C with the lactose clusters ($1 \mu\text{M}$). Cells were subsequently incubated in the presence (black bars) or absence (white bars) of monensin at 4 °C, and fluorescence intensity was assessed by flow cytometry. $K_n\text{Lac}_{n+1}$ represents $\text{Lac}\beta\text{-Glp-}\beta\text{Ala}[\text{Lys}(\text{Lac}\beta\text{-Glp-}\beta\text{Ala})_n\text{Ala-Cys(Flu)-NH}_2$, with $n = 1$ to 5; $(KA)_5\text{Lac}_6$ represents **5f**, and $K_4\text{Man}_6\text{Man}_5$ represents **6d** with dimannose. (B) A representative experiment of the uptake of oligokisdimannose clusters by DΦ cells. DΦ cells (2×10^5) were incubated for 2 h at 37 °C with the dimannose $\text{Man}\alpha 6\text{Man}$ clusters ($1 \mu\text{M}$). Cell fluorescence intensity was assessed by flow cytometry, after subsequent incubation at 4 °C with (black bars) or without (white bars) monensin. $K_n\text{Os}_{n+1}$ represents $\text{Man}\alpha 6\text{Man}\beta\text{-Glp-}\beta\text{Ala}[\text{Lys}(\text{Man}\alpha 6\text{Man}\beta\text{-Glp-}\beta\text{Ala})_n\text{Ala-Cys(Flu)-NH}_2$, $n = 1$ to 5, and $K_4\text{Lac}_5$ represents **5d**.

had a higher affinity for ConA than the two other clusters synthesized from $\text{Man}\alpha 3\text{Man}\beta\text{-Glp-}\beta\text{Ala}$ and $\text{Man}\alpha 6\text{Man}\beta\text{-Glp-}\beta\text{Ala}$, which have a low affinity (Table 3).

Endocytosis of oligolysine clusters

The endocytosis efficiency of two series of lysine-based disaccharide clusters, containing up to five lysine residues, was assessed by flow cytometry using two types of cells, HepG2 cells and DΦ dendritic cells; three or more independent experiments were performed and one typical experiment is presented. The endocytosis of lactose clusters by HepG2 cells (Figure 4A) was poor with bis- and tris-lactose clusters, $K_1\text{Lac}_2$, $K_2\text{Lac}_3$ respectively, and large with tetrakis- and pentakis-lactose clusters, $K_3\text{Lac}_4$ and $K_4\text{Lac}_5$ respectively. The endocytosis was maximal with both hexakis-lactose clusters based on either a pentalysine derivative ($K_5\text{Lac}_6$) or a pentalysylalanine derivative ($\text{Lac}[K(\text{Lac})\text{A}]_5$), showing that the presence of an intercalating amino acid between two ϵ -glycosylated lysines did not change the recognition efficiency.

Endocytosis of the dimannose ($\text{Man}\alpha 6\text{Man} = \text{Os}$) cluster by DΦ dendritic cells (Figure 4B) was very poor with bis- and tris-dimannose clusters ($K_1\text{Os}_2$ and $K_2\text{Os}_3$ respectively), suboptimal with tetrakis-dimannose cluster ($K_3\text{Os}_4$) and maximal with both pentakis- and hexakis-dimannose clusters ($K_4\text{Os}_5$ and $K_5\text{Os}_6$ respectively).

Figure 5 Uptake of pentakisdimannose clusters by DΦ cells

DΦ cells were incubated at 37 °C for 2 h with the pentakisdimannose clusters, and after subsequent incubation with (black bars) or without (white bars) monensin at 4 °C, fluorescence intensity was assessed by flow cytometry. X represents $\alpha 6$, $\alpha 3$ or $\alpha 2$.

The endocytosis specificity with regards to the cell type was clearly demonstrated by showing that optimal lactose clusters were efficiently taken up by HepG2 cells and not at all by DΦ dendritic cells, and conversely that optimal dimannose clusters were efficiently taken up by DΦ dendritic cells and quite poorly by HepG2 cells (Figures 4A and 4B).

Upon endocytosis, disaccharide clusters were localized in acidic compartments, as shown by a cell-associated fluorescence increase upon incubation at 4 °C in the presence of 50 μM monensin (Figures 4A and 4B). Indeed, monensin is a proton/sodium ionophore which transports univalent cations (Na^+ and H^+) across membranes and, thereby, equilibrates the external and internal pH of organelles in intact cells [14]. Thus, the enhancement of the cell fluorescence intensity upon monensin treatment is due to the neutralization of endosomes and lysosomes. It is worthy of note that the monensin-dependent enhancement was much larger in the case of HepG2 cells than in the case of DΦ dendritic cells, reflecting the fact that the fluorescent clusters were delivered into more acidic compartments in the hepatoma cells than in dendritic cells.

Dimannose clusters were taken up with an efficiency which partially depended on the linkage between the two mannoses (Figure 5): $\text{Man}\alpha 6\text{Man}$ dimannose cluster uptake was the most efficient, whereas $\text{Man}\alpha 2\text{Man}$ dimannose cluster uptake was the least efficient.

DISCUSSION

Cells express on their surface a variety of sugar receptors called lectins (reviewed in [23,24]). These cell surface lectins, as well as their ligands, sugar moieties of specific glycoconjugates, are involved in several important phenomena, including cell-cell recognition, glycoconjugate-cell recognition and the uptake of glycoconjugates. This last property is the basis of glycotargeting (reviewed in [25–27]). Dendritic cells, which are key cells for antigen presentation and cellular vaccination (reviewed in [28]), express mannose-specific lectins [9,10,29] and are therefore suitable targets for drug delivery based on glycoconjugates containing mannose.

The recognition of a simple sugar by a lectin is usually in the very low affinity range (approx. $10^3 \text{ litre} \cdot \text{mol}^{-1}$). Conversely,

complex oligosaccharides, neoglycoproteins, as well as saccharide clusters, bind lectins in the high affinity range (up to 10^7 – 10^8 litre $\cdot$ mol $^{-1}$; reviewed in [18,19]). For clinical purposes, animal glycoproteins including neoglycoproteins should be avoided because they may be contaminated by virus or prions etc., while synthetic carbohydrate clusters are suitable. Indeed, several authors have developed different glycoclusters (reviewed in [19,30,31], including glycodendrimers, glycostarburst, glycostarfish and glycosylated peptides. Glycoclusters may be synthesized using classical chemical approaches starting with fully protected sugars (reviewed in [32]).

With the aim of creating therapeutic tools for targeting oligonucleotides and genes, we designed glycoclusters that are made of natural metabolites, namely amino acids and sugars. Glycoclusters are made of glycosynthons [6] which are prepared according to a one-pot two-step reaction, starting with reducing sugars and a glutamate derivative. In the present paper, we used Glu- β -Ala-benzyl ester instead of Glu-*p*-nitroanilide [21] in order to get rid of the anilide moiety. Such glycosynthons, after benzyl group removal, were activated and linked to the amino groups of an oligolysine, in the presence of molecular sieves. Molecular sieves allow an increase in the reaction yield from approx. 60% amino group substitution [7] up to completion. The use of oligolysine to prepare glycoclusters was pioneered by Ponpipom [1] and Robbins [4] and used by others including Toyokuni and Hakomori [33], as well as Biessen and co-workers [5,34], and Grandjean and co-workers [3,35]. The preparation of the glycoclusters presented in the present paper did not require any protection of the hydroxyl groups of the sugar moiety, in contrast with Ponpipom [1], Kragol [36] or Kichler [2], who used peracetylmannosylthiopropionate, peracetylmannosylserine or peracetylgalactosylthioethoxyethyl derivatives respectively. The yield of glycoclusters using the preparation described in the present paper was quite high, up to 90% for the tetrakis dimannosyl trilyserine or pentakis lactose tetralysine, in contrast with yields of 34% for the tetrakis mannose trilyserine [5] using monosaccharyl phenylisothiocyanate. The method used in the present paper allows the preparation of glycoclusters containing disaccharide as well as oligosaccharides (results not shown); the unique requirement is to use an oligosaccharide which terminates with a reducing sugar as the starting material. The possibility of using disaccharides or higher oligosaccharides was thought to enhance the selectivity. Indeed, plant and animal lectins recognize disaccharides and oligosaccharides more selectively than simple sugars. For instance, the macrophage mannose-specific lectin recognizes an oligomannoside containing a Man α 6Man α motif more efficiently than a monosaccharide [37]. Knowing that natural glycans contain Man α 2Man, Man α 3Man and Man α 6Man, we prepared glycoclusters containing these disaccharides, as well as glycoclusters containing lactose as a control.

The association constants for a series of lactose clusters and a series of dimannose clusters were deduced from SPR experiments. In most of the published data, the interactions are measured by running the lectin on immobilized oligosaccharides. We have chosen immobilization of the lectin in order to compare easily the affinities of a series of different glycoconjugates, and also because Shinohara and co-workers [38] showed that when the lectin was immobilized, the calculated affinity constant was in good agreement with values obtained using classical methods with lectins in solution. The analysis of the cluster binding to plant lectins indicated that Rca, a galactose-specific lectin, recognized lactose clusters, and amongst them, the pentakis lactose tetralysine with a high affinity, while it did not recognize any dimannose clusters. Similarly, ConA did not recognize the lactose clusters but it did bind the dimannose clusters, the best ligand

being the cluster made of Man α 2Man, in agreement with published data (reviewed in [39,40]).

The glycoclusters as fluorescence-labelled derivatives were shown to be specifically recognized and taken up by cells expressing membrane lectins. HepG2 cells, which express a galactose-specific lectin [8,41] but not a mannose-specific lectin, optimally took up clusters containing at least four lactose groups, but failed to take up any dimannose clusters. Similarly, D Φ dendritic cells, which express mannose-specific lectins, a macrophage mannose-specific receptor (reviewed in [42]), as well as dendritic cell-specific ICAM-grabbing non-integrin (DC-SIGN, [29]), optimally recognized and took up glycoclusters containing five dimannoses, whereas they failed to recognize lactose clusters. In addition, the dimannose clusters containing Man α 6Man were more efficiently taken up by D Φ dendritic cells than those containing either Man α 3Man or Man α 2Man.

The preferential uptake of Man α 6Man clusters by dendritic cells is in agreement with the fact that oligomannosides containing Man α 6Man are the best ligands for the macrophage-like mannose receptor [37]. This selective uptake may mean either that DC-SIGN prefers the Man α 6Man based clusters, or that it is not predominantly involved in the uptake of any dimannoside clusters. This last hypothesis could be in accord with the recent results published [43], which showed, on the basis of three-dimensional studies, that the binding site of DC-SIGN accommodates high-mannose oligomannosides containing branched structures such as Man α 6(Man α 3)Man α .

CONCLUDING REMARKS

The conditions for the preparation of well-defined sugar clusters based on disaccharides and an oligolysine core have been optimized; their binding capacity is optimal when five sugar moieties are present. The use of such sugar clusters as recognition signals for antigen and/or gene targeting in a vaccine approach, as well as the preparation and the use of glycoclusters containing glycosynthons made with three and more sugar units, are currently being investigated in our laboratory.

We thank C. Buré, P. O. Schmit, M. T. Bousser and Y. Hachad for their skillful help with ESI, MALDI, biological tests on cells and SPR analysis respectively. BIAcore 2000 and FACS sort were purchased using grants from ARC (Association de Recherche contre le Cancer) and a University of Orléans grant from the Région Centre Council. We thank C. Bocaccio and J. P. Abastado (IDM, Paris) for kindly providing the dendritophagocytic cells. This work was partly supported by grants from Association Française de Lutte contre la Mucoviscidose (AFLM). N.F. received a fellowship from the Région Centre Council. A.C.R. is Research Director at INSERM. M.M. is Emeritus Professor at the University of Orléans. R.M. is Research Director at CNRS.

REFERENCES

- Ponpipom, M. M., Bugianesi, R. L., Robbins, J. C., Doebber, J. C. and Shen, T. Y. (1981) Cell-specific ligands for selective drug delivery to tissues and organs. *J. Med. Chem.* **24**, 1388–1395.
- Kichler, A. and Schuber, F. (1995) Versatile synthesis of bi- and tri-antennary galactose ligands: interaction with the Gal/GalNAc receptor of human hepatoma cells. *Glycoconj. J.* **12**, 275–281.
- Grandjean, C., Rommens, C., Gras-Masse, H. and Melnyk, O. (2000) One-pot synthesis of antigen-bearing, lysine-based cluster mannosides using two orthogonal chemoselective ligation reactions. *Angew. Chem. Int. Ed.* **39**, 1068–1072.
- Robbins, J. C., Lam, M. H., Tripp, C. S., Bugianesi, R. L., Ponpipom, M. M. and Shen, T. Y. (1981) Synthetic glycopeptide substrates for receptor-mediated endocytosis by macrophages. *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7294–7298.
- Biessen, E. A. L., Noorman, F., van Teijlingen, M. E., Kuiper, J., Barrett-Bergshoeff, M., Bijsterbosch, M. K., Rijken, D. C. and van Berkel, T. J. (1996) Lysine-based cluster mannosides that inhibit ligand binding to the human mannose receptor at nanomolar concentration. *J. Biol. Chem.* **271**, 28024–28030.

- 6 Sdiqui, N., Roche, A. C., Mayer, R. and Monsigny, M. (1995) New synthesis of glycopeptides. *Carbohydr. Lett.* **1**, 269–275
- 7 Bédouet, L., Bousser, M. T., Frison, N., Boccaccio, C., Abastado, J. P., Marceau, P., Mayer, R., Monsigny, M. and Roche, A. C. (2001) Uptake of dimannoside clusters and oligomannosides by human dendritic cells. *Biosci. Rep.* **21**, 839–855
- 8 Schwartz, A. L., Fridovich, S. E., Knowles, B. B. and Lodish, H. F. (1981) Characterization of the asialoglycoprotein receptor in a continuous hepatoma line. *J. Biol. Chem.* **256**, 8878–8881
- 9 Avrameas, A., McIlroy, D., Hosmalin, A., Autran, B., Debre, P., Monsigny, M., Roche, A. C. and Midoux, P. (1996) Expression of a mannose/fucose membrane lectin on human dendritic cells. *Eur. J. Immunol.* **26**, 394–400
- 10 Engering, A. J., Cella, M., Fluitsma, D., Brockhaus, M., Hoefsmit, E. C., Lanzavecchia, A. and Pieters, J. (1997) The mannose receptor functions as a high capacity and broad specificity antigen receptor in human dendritic cells. *Eur. J. Immunol.* **27**, 2417–2425
- 11 Roche, A. C., Barzilay, M., Midoux, P., Junqua, S., Sharon, N. and Monsigny, M. (1983) Sugar-specific endocytosis of glycoproteins by Lewis lung carcinoma cells. *J. Cell Biochem.* **22**, 131–140
- 12 Monsigny, M., Petit, C. and Roche, A. C. (1988) Colorimetric determination of neutral sugars by a resorcinol sulfuric acid micromethod. *Anal. Biochem.* **175**, 525–530
- 13 Monsigny, M., Roche, A. C. and Midoux, P. (1984) Uptake of neoglycoproteins via membrane lectin(s) of L1210 cells evidenced by quantitative flow cytometry and drug targeting. *Biol. Cell* **51**, 187–196
- 14 Midoux, P., Roche, A. C. and Monsigny, M. (1987) Quantitation of the binding, uptake, and degradation of fluoresceinylated neoglycoproteins by flow cytometry. *Cytometry* **8**, 327–334
- 15 Langmuir, I. (1918) The adsorption of gases on platine surfaces glass mica and platinum. *J. Am. Chem. Soc.* **40**, 1361–1403
- 16 Scatchard, G. (1949) The attraction of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51**, 660–672
- 17 Nisonoff, A. and Pressman, D. (1958) Heterogeneity and average combining constants of antibodies from individual rabbits. *J. Immunol.* **80**, 417–428
- 18 Lee, Y. C. and Lee, R. T. (1994) Enhanced biochemical affinity of multivalent neoglycoconjugates. In *Neoglycoconjugates: preparation and application* (Lee, Y. C., ed.), pp. 23–50, Academic Press, San Diego
- 19 Monsigny, M., Mayer, R. and Roche, A. C. (2000) Sugar-lectin interactions: sugar clusters, lectin multivalency and avidity. *Carbohydr. Lett.* **4**, 35–42
- 20 Sips, R. (1948) Structure of catalyst surface. *J. Chem. Phys.* **16**, 490–495
- 21 Quétyard, C., Bourgerie, S., Normand-Sdiqui, N., Mayer, R., Strecker, G., Midoux, P., Roche, A. C. and Monsigny, M. (1998) Novel glycosynthons for glycoconjugate preparation: oligosaccharylpyroglutamylamide derivatives. *Bioconjug. Chem.* **9**, 268–276
- 22 Ohsumi, Y., Hoppe, C. A., Ogawa, T. and Lee, Y. C. (1988) Enhancement of macromolecular ligand binding by rabbit alveolar macrophages by mannose oligosaccharides and related compounds. *Arch. Biochem. Biophys.* **260**, 241–249
- 23 Lis, H. and Sharon, N. (1998) Lectins: carbohydrate-specific proteins that mediate cellular recognition. *Chem. Rev.* **98**, 637–674
- 24 Varki, A., Cummings, R., Esko, J., Freeze, H., Hart, G. and Marth, J. (eds.) (1999) *Essentials of Glycobiology*, Cold Spring Harbor Laboratory Press, New York
- 25 Monsigny, M., Roche, A. C., Midoux, P. and Mayer, R. (1994) Glycoconjugates as carriers for specific delivery of therapeutic drugs and genes. *Adv. Drug Delivery Rev.* **14**, 1–24
- 26 Wadhwa, M. S. and Rice, K. G. (1995) Receptor mediated glycotargeting. *J. Drug Target.* **3**, 111–127
- 27 Monsigny, M., Midoux, P., Mayer, R. and Roche, A. C. (1999) Glycotargeting: influence of the sugar moiety on both the uptake and the intracellular trafficking of nucleic acid carried by glycosylated polymers. *Biosci. Rep.* **19**, 125–132
- 28 Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., Pulendran, B. and Palucka, K. (2000) Immunobiology of dendritic cells. *Annu. Rev. Immunol.* **18**, 767–811
- 29 Geijtenbeek, T. B., Torensma, R., van Vliet, S. J., van Duinhoven, G. C., Adema, G. J., van Kooyk, Y. and Figdor, C. G. (2000) Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell* **100**, 575–585
- 30 Roy, R. (1996) Syntheses and some applications of chemically defined multivalent glycoconjugates. *Curr. Opin. Struct. Biol.* **6**, 692–702
- 31 Jayaraman, N., Nepogodiev, S. A. and Stoddart, J. F. (1997) Synthetic carbohydrate-containing dendrimers. *Chem. Eur. J.* **3**, 1193–1199
- 32 Danishefsky, S. J. and Allen, J. R. (2000) From the laboratory to the clinic: a retrospective on fully synthetic carbohydrate-based anticancer vaccines. *Angew. Chem. Int. Ed.* **39**, 836–863
- 33 Toyokuni, T. and Hakomori, S. I. (1994) Carbohydrate-lysyllysine conjugates as cell antiadhesion agents. *Methods Enzymol.* **247**, 325–341
- 34 Biessen, E. A., Beuting, D. M., Roelen, H. C., van de Marel, G. A., van Boom, J. H. and van Berkel, T. J. (1995) Synthesis of cluster galactosides with high affinity for the hepatic asialoglycoprotein receptor. *J. Med. Chem.* **38**, 1538–1546
- 35 Grandjean, C., Gras-Masse, H. and Melnyk, O. (2001) Synthesis of clustered glycoside-antigen conjugates by two one-pot, orthogonal, chemoselective ligation reactions: scope and limitations. *Chemistry* **7**, 230–239
- 36 Kragol, G. and Otvos, J. (2001) Orthogonal solid-phase synthesis of tetramannosylated peptide constructs carrying three independent branched epitopes. *Tetrahedron* **57**, 957–966
- 37 Maynard, Y. and Baenziger, J. U. (1981) Oligosaccharide specific endocytosis by isolated rat hepatic reticuloendothelial cells. *J. Biol. Chem.* **256**, 8063–8068
- 38 Shinohara, Y., Kim, F., Shimizu, M., Goto, M., Tosu, M. and Hasegawa, Y. (1994) Kinetic measurement of the interaction between an oligosaccharide and lectins by a biosensor based on surface plasmon resonance. *Eur. J. Biochem.* **223**, 189–194
- 39 So, L. L. and Goldstein, I. J. (1968) Protein-carbohydrate interaction. 13. The interaction of concanavalin A with alpha-mannans from a variety of microorganisms. *J. Biol. Chem.* **243**, 2003–2007
- 40 Goldstein, I. J. and Poretz, R. D. (1986) Isolation, physicochemical characterization, and carbohydrate-binding specificity of lectins. In *The lectins. Properties, functions, and applications in biology and medicine* (Liener, I. E., Sharon, N. and Goldstein, I. J., eds.), pp. 33–247, Academic Press, San Diego
- 41 Ashwell, G. and Harford, J. (1982) Carbohydrate-specific receptor of the liver. *Annu. Rev. Biochem.* **51**, 531–554
- 42 Stahl, P. D. and Ezekowitz, R. A. (1998) The mannose receptor is a pattern recognition receptor involved in host defense. *Curr. Opin. Immunol.* **10**, 50–55
- 43 Feinberg, H., Mitchell, D. A., Drickamer, K. and Weis, W. I. (2001) Structural basis for selective recognition of oligosaccharides by DC-SIGN and DC-SIGNR. *Science* **294**, 2163–2166

Received 26 April 2002/9 July 2002; accepted 15 July 2002

Published as BJ Immediate Publication 15 July 2002, DOI 10.1042/BJ20020673