Carbohydrates in an Acidic Multivalent Assembly: Nanomolar P-Selectin Inhibitors

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Neutrophil recruitment to tissues is initiated by an adhesion cascade.[1] Through this process, cells roll and eventually attach firmly to the endothelium.[2−9] The factors that contribute to the high binding strength of this interaction are not well understood. Here we report that multivalent carbohydrate assemblies can mimic neutrophils with respect to adhesion. However, the multiplicity of sugar copies in itself is not sufficient to provide tight binding. The chemical constituents making up the underlying backbone play an important, if not greater, role in this interaction. This is demonstrated by the nanomolar inhibition of adhesion by a simple sugar (lactose) presented on the surface of an acidic, polymerized liposome. Since various diseases such as ARDS (adult respiratory distress syndrome), rheumatoid arthritis, septic shock, and reperfusion injury can result from neutrophil adhesion, polymerized liposomes presenting carbohydrates (glycoliposomes) may form the basis for a new therapeutic agent.

A physiological ligand for the adhesion protein P-selectin has been recently identified as a mucin-like structure designated as PSGL-1 (P-selectin glycoprotein ligand 1).[10,11] Some sialylated or sulfated fucooligosaccharides, for example sialyl Lewis^x (sLe^x, **1**) (Figure 1), exhibit weak binding to P-selectin.[4,12−14] Although much evidence suggests that the PSGL-1 carbohydrate epitope is an sLe^x-type structure, it is apparent that other factors are required for the high, *in vivo* affinity of P-selectin.[2,15−22]

We have designed macromolecular structures capable of potent inhibitory activity toward P-selectin binding. Polymerizable, carbohydrate-terminated lipid monomers are readily assembled into liposomes.[23] These structures are made stable by polymerization with UV light. Nanomolar P-selectin inhibition is observed when liposomes are prepared from a bicomponent mixture of sLe^x-like glycolipids and acidic lipids.

The polymerizable glycolipid **2b** was synthesized by coupling the *N*-glycoside of compound **2a** to commercially available 10,12-pentacosadiynoic acid (PDA, **5**) (Figure 1).[24] Compound **5** (PDA) or **6** is employed as the "matrix" lipid in the liposome preparations. The glycolipids are mixed with the matrix lipids and are formed into glycoliposomes by the probe sonication method[25] and polymerized by UV irradiation (254 nm).[26] Compounds **5** and **6** give the polymer scaffold an acidic (**5**) or neutral (**6**) character and separate the large carbohydrate headgroups on the bilayer surface (Figure 2).

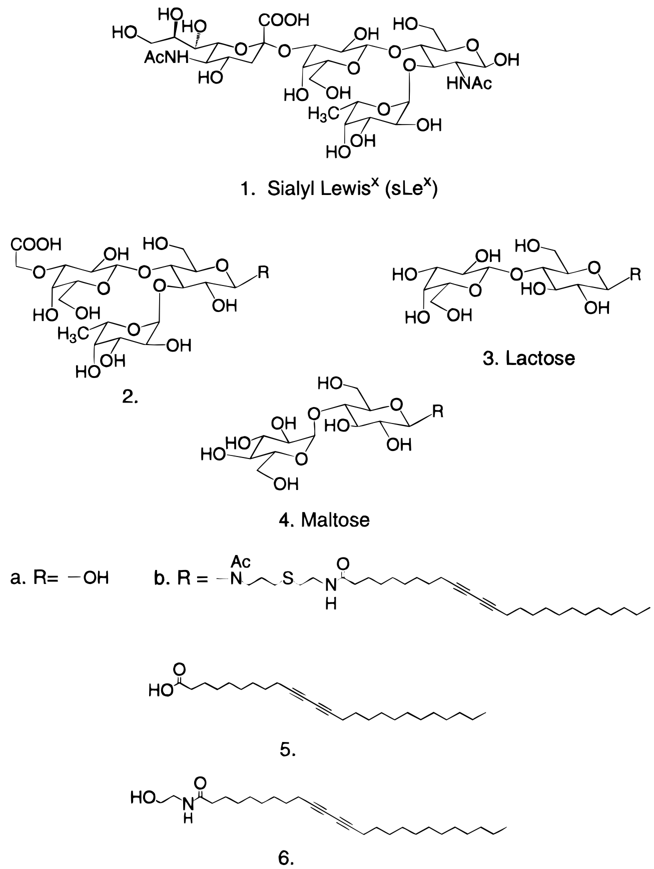

Figure 1. The sLe^x tetrasaccharide (**1**) recognized by P-selectin. Carbohydrates **2a** (sLe^x analog), **3a** (lactose), and **4a** (maltose) are used for synthesizing the polymerizable glycolipids **2b**, **3b**, and **4b**, respectively. Compound **5** or **6** is used as the matrix lipid in liposome preparation.

Glycoliposomes were prepared with acidic matrix lipid **5** and varying percentages of glycolipid **2b** in the range 0−50%. Activity was determined by assaying for inhibition of P-selectin IgG chimera binding to HL-60 cells.[12] The glycoliposomes showed a dose dependent inhibition (shown in Figure 3A). The inhibition is highly dependent on the percentage of glycolipid in the glycoliposomes. Concentrations of approximatly 5 molar % glycolipid **2b** gave the best inhibition (IC_{50} = 2 nM) that diminished with higher or lower surface concentrations (shown in Figure 3B). Glycoliposomes prepared from lactose or maltose glycolipids (**3b** or **4b**, with matrix lipid **5**) also showed binding inhibition (Figure 3A). The IC_{50} values for lactose glycoliposomes (**3b**) is 7 times weaker, and maltose glycoliposomes (**4b**) is 2 orders of magnitude weaker, than the sLe^x analog (**2b**) (Figure 3C). The same trend with respect to surface percentages vs activity is observed for all glycoliposomes tested (data not shown). These IC_{50} values are in stark contrast to the monomeric carbohydrate **2a**, **3a**, or **4a**. The monomeric IC_{50} value for **2a** is greater than 5 mM[4] and lactose or maltose have no observed binding.

In contrast, glycoliposome preparations with 5% **2b** in neutral matrix lipid **6** showed no inhibitory activity at the highest concentration tested, 500 nM (Figure 3A). Therefore, the acidic groups of matrix lipid **5** are essential for strong binding activity. This result correlates with the apparent requirement for anionic groups in P-selectin antagonists. For example, the known inhibitors heparin, inositol hexakisphosphate,

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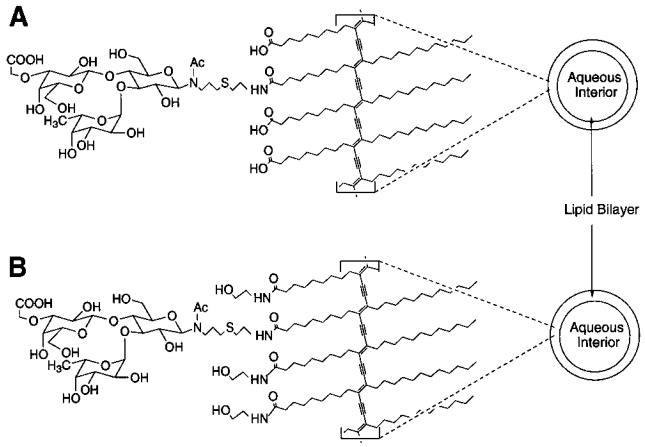

Figure 2. Schematic of outer monolayer in a polymerized glycoliposome bilayer containing compounds **2b** and **5** (A) or **2b** and **6** (B). The various molar percentages of lipids are mixed and formed into liposomes. Samples were prepared so as to give 1 mM solutions in total lipid while varying the percentages of glycolipids in the range 0.5−50%. The lipids appear to be miscible based on an analysis of their Langmuir isotherms.[38] Therefore, the reported carbohydrate percentages are estimates of the sugar groups appearing on both the inner and outer liposome surfaces. Polymerization of the liposomes was carried out by exposure of the aqueous solutions to UV light.[23] Polymerization of lipid diacetylenes requires the monomers to adopt a solid analogus state. With percentages of the glycolipid component above approximately 40%, polymerization was substantially inhibited. This is rationalized by the steric crowding of adjacent carbohydrate headgroups which prevent the proximal diacetylenes from polymerizing. Characterization of the polymerized glycoliposomes by transmission electron microscopy (TEM) shows spheres between 20 and 100 nm in diameter.

sulfoglucuronyl glycolipids, fucoidan, sulfatides, and an sLe^x−RGD conjugate are all carbohydrate and carbohydrate-like molecules with multiple acid groups.[27−31] Also, recent evidence indicates that there is an essential acidic group in the form of a sulfated tyrosine residue in the peptide backbone of PSGL-1.[32−34] However, since the liposomes prepared from 100% acidic lipid **5** did not show inhibitory activity, both the carbohydrate and acidic lipid functionalities are needed for activity (Figure 3C). This concurs with the results seen for PSGL-1. To produce a high-affinity ligand, tyrosine sulfation alone is insuffient; these acidic sites must work in concert with juxtapositioned oligosaccharide groups.[33]

These results support the findings that P-selectin (unlike E-selectin) binds to a range of carbohydrates with varying affinities.[14,15,35] High-affinity P-selectin inhibitors can be prepared from polymerized glycoliposomes with an acidic matrix lipid. Both the carbohydrate and the acidic matrix lipid are essential for binding. The finding that lactose and maltose glycoliposomes bind to P-selectin demonstrates that these carbohydrates, even if they are devoid of any activity in the monomeric forms, are capable of showing considerable activity as multivalent arrays when presented in conjunction with a charged scaffold. On the basis of this study, it cannot be ruled out that the glycoliposomes containing lactose and maltose might bind to an alternative site on P-selectin which also recognizes polyanionic portions and carbohydrate determinants other than sLe^x. Studies are now ongoing in an effort to elucidate the carbohydrate and surface properties necessary to effect differential inhibition of all three selectins (E, L, and P).

Polysaccharide coated liposomes have been used *in vivo* to target specific cells or tissues.[36] Due to recent advances in liposome formulations (resulting in vesicles coated by either monosialogangliosides GM_1 or lipid derivatives of polyethylene glycol), liposomes that have long circulation half-lives have been realized.[37] *In vivo* studies with carbohydrate expressing, polymerized lip-

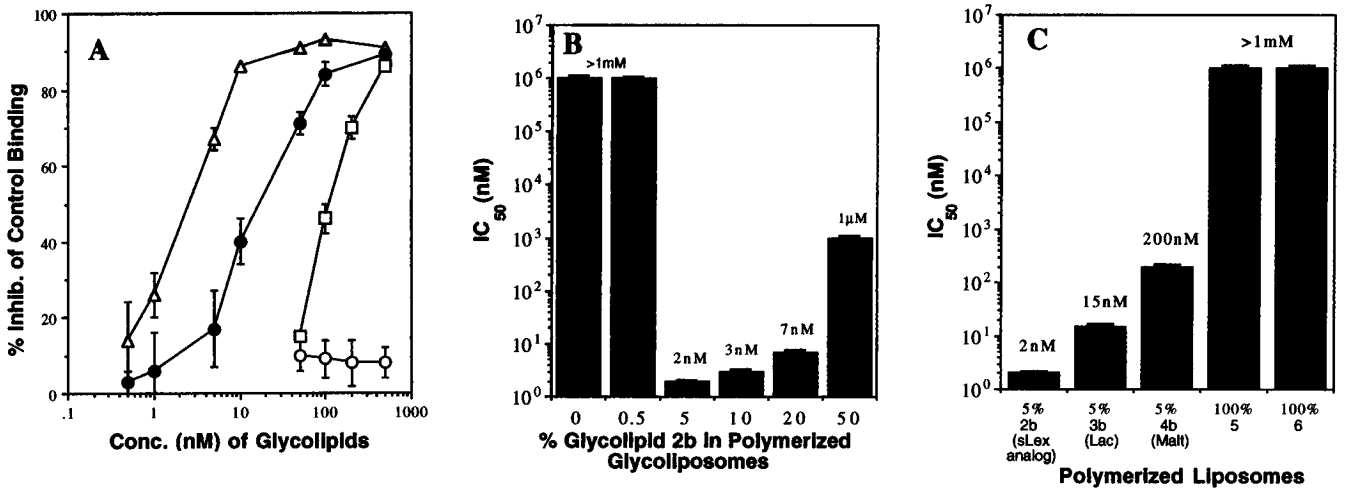

Figure 3. Inhibition potencies of polymerized glycoliposomes toward P-selectin IgG chimera binding to HL-60 cells. (A) The effect of serial dilutions of the glycoliposomes containing 5% **2b** (sLe^x analog) with acidic matrix lipids **5** (△) or neutral matrix lipids **6** (○); glycoliposomes containing 5% **3b** (lactose lipid) (●) and 5% **4b** (maltose lipid) (□) both with acidic matrix lipids **5** on the inhibition of P-selectin. (B) IC_{50} values of glycoliposomes containing glycolipid **2b** and matrix lipid **5** verses the percentage of this glycolipid in the polymerized liposomes. (C) The relative IC_{50} values of glycoliposomes containing glycolipids **2b**, **3b**, and **4b** (made with matrix lipid **5**) and the polymerized liposomes containing only matrix lipids **5** and **6**. For the glycoliposomes, the IC_{50} values are based on the total concentration of glycolipid. No assumption was made as to the amount of glycoside that may be inaccessible due to incorporation into the inner layer of the liposome. Therefore, these IC_{50} values represent an upper limit of the actual glycoside available for binding. The inhibitor activity was determined in a competitive adhesion assay with HL-60 cells, according to the described protocol.[12] Briefly, P-selectin chimera is allowed to form a complex with biotinylated goat F(ab′), antihuman IgG Fc, and alkaline phosphatase-streptavidin and is preincubated with inhibitors before mixing with HL-60 cells. The cells were pelleted by centrifugation and washed with TBS. Chromagen was added, and the color that developed was read as an OD at 405 nm. All assays were run in quadruplicate. The possibility of intercalation of the liposomes into the cells, thereby effecting their ability to bind P-selectin, was addressed. The cells were pretreated with the liposomes and washed to remove the liposomes prior to the addition of the P-selectin chimera. This did not result in any reduction in selectin binding to the cells. The inhibition was unaffected in experiments where the reagents and inhibitors were added simultaneously to the microtiter plates.

osomes may lead to their utilization in anti-inflammation therapies.

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