Allylmalonamide as a bivalent linker: Synthesis of biantennary GM₃-saccharide—Keyhole limpet hemocyanin glycoconjugate and the immune response in mice†

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A biantennary GM₃-saccharide (sialyllactoside) derivative (4) was constructed using allylmalonic acid as a bivalent linker, both carboxylic acids of which were condensed with 3-aminopropyl lactoside (2) prior to enzymatic sialylation with a fusion enzyme. While ozonolysis of its allyl group generated a saccharide having a terminal aldehyde (6), we were unable to couple 6 directly to protein by reductive amination. However, extension of the spacer by means of introducing a maleimide group to 6 through its aldehyde group to give 7 enabled the latter to be successfully coupled to thiolated proteins. The average ratios of saccharide to protein were observed to be 35 in KLH conjugate (13) and 9–12 in HSA conjugates (14 and 15). The antisera obtained by immunizing mice with the biantennary sialyllactoside-KLH conjugate (13) together with MPL adjuvant were analyzed by ELISA. Using several structurally related saccharide-HSA conjugates as screening antigens, it was concluded that anti-sialyllactoside antibodies, both IgG and IgM, were effectively raised. This was further supported by competitive inhibition experiments using lactoside (1), sialyllactoside (8) and biantennary sialyllactoside (4) as inhibitors.

Keywords: GM₃ antigen, sialyllactoside, biantennary, glycoconjugate, antibody

Abbreviation: CTP, Cytidine 5'-triphosphate; KLH, Keyhole Limpet Hemocyanin; M₄C₁₉H, 4-(4-N-maleimidomethyl) cyclohexane-1-carboxyl hydrazide; MPL, Monophosphoryl lipid A; Sulfo-GMBS, N-(γ-maleimidobutyloxy) sulfosuccinimide ester; BSA, Bovine serum albumin; HSA, Human serum albumin; GBSPla (GBSPIII), Type la (III) group B Streptococcus polysaccharide

Introduction

Gangliosides including GM₁ are over-expressed on the surface of several human tumors, particularly melanomas [1–4], although GM₃ is not unique to melanomas as it is also found on normal human cells, but at much lower density. However, a murine IgM mAb (M2590) was reported to react with GM₃ antigen on B16 melanoma but not on normal cells. This was attributed to differences in the density and tertiary structure of GM₃-saccharides on the surface of these cells [5,6]. In addition a GM₃-enriched microdomain in B16 melanoma has been reported to be involved in cell adhesion and signal transduction [7], which could be important in promoting metastasis.

The humoral immune response to specific gangliosides on tumor cells plays an important role in host protective immunity and correlates with prolonged survival [8–11], however, GM₃, unlike other gangliosides, is incapable of inducing an antibody response [12] because of its immune suppressive role [13,14]. Considerable effort has been devoted in trying to raise anti-GM₃ antibodies, particularly IgG, by using various adjuvants, but this has met with limited success [15–17]. Only when GM₃ lactone was used were IgG antibodies raised, which cross-reacted with GM₃ and inhibited the melanoma cell growth [18].

Improvement in the immunogenicity of ganglioside conjugate vaccines was obtained using KLH as a carrier [19,20]. This was particularly true for GD₂ and GM₂ in terms of both antibody production and host protection [21,22]. Because of the high concentration of GM₁ on the tumor cell surface we hypothesized that synthetic glycoconjugates having a multivalent rather than monovalent presentation of GM₃ would be more successful in triggering immune response by raising tumor specific antibodies.
Therefore, as the first step in the assembly of multiantennary GM3-saccharide motifs coupled to KLH we describe the synthesis of a KLH glycoconjugate of a biantennary GM3-saccharide, and preliminary studies on its immune response in mice.

Materials and Methods

General methods

$^1$H and $^{13}$C NMR spectra were recorded at 500 MHz and 125 MHz, respectively, with INOVA-500 instrument at 293 K unless otherwise noted. Chemical shifts are given in ppm relative to the signal of internal acetone $\delta_H$ 2.225 in D$_2$O for $^1$H NMR spectra, and to $\delta_C$ 31.07 for $^{13}$C NMR spectra. The $^1$H NMR chemical shifts of oligosaccharides were assigned on the basis of 2D $^1$H-COSY and $^1$H-$^{13}$C chemical-shift correlated experiments. ES-MS were performed with QUATTRO (MICROMASS). MALDI-MS spectra were recorded with Voyager-DE™ STR (PerSeptive Biosystems). HPLC analysis was performed with an instrument of Hewlett Parkard Series 1100 using a Superose 12 10/30 column (Pharmacia), PBS buffer was used as eluent. Both UV and RI monitors were equipped.

KLH and maleimide crosslinkers (Sulfo-GMBS and M$_2$C$_2$H) were the products of PIERCE. HSA was a product of Sigma. All other chemicals were purchased from Aldrich without further purification. Two glycoconjugates (GBSPIa-HSA and GBSPIII-HSA) were prepared with the procedure previously reported [23].

Synthesis of biantennary sialyllactosyl-KLH conjugate

3-Aminopropyl lactoside (2). A solution of 1 (0.5 g) in water (5 ml) was added Pd/C (100 mg, 50% wet). The mixture was subjected to hydrogen pressure (35 p.s.i.) for 2 h, when TLC indicated the reaction was complete. The filtrate was passed through a Sephadex G-10 column, using water as eluent. The fractions were pooled and lyophilized to afford 2 (0.43 g, 90%) as an amorphous. Calcd for C$_{15}$H$_{29}$NO$_{11}$: 414.4. Found: 415.3 (M$^+$ positive).

N,N′-Di-sialyllactosylpropyl allylmalonamide (3). A solution of 2 (100 mg, 0.25 mmole) in DMF (0.5 ml) were added iPr$_2$NEt (40 μL) and a solution of pentafluorophenyl allylmalonate (50 mg, 0.11 mmole) in DMF (0.25 ml). The mixture was kept at room temperature for 4 h and precipitated by the addition of diethyl ether. The pellet was dissolved in water and purified on a Biogel P-2 column using water as eluent to obtain a white solid 3 (65 mg, 65%) after lyophilize as major product. Calcd for C$_{36}$H$_{62}$N$_2$O$_{24}$: 906.9. Found: 907.2 (M$^+$ H$^-$) (f.a.b. positive); 905.4 (M$^+$ H$^-$) (ES-MS negative).

N,N′-Di-(sialyllactosyl)propyl allylmalonamide (4) and N-lactosylpropyl-N′-(sialyllactosyl) propyl allylmalonamide (5). A solution of 3 (27 mg) in water (20 ml) were added 0.1M MgCl$_2$ (2 ml), 0.1 M NeuAc (1 ml), and 0.1 M CTP (1 ml) and adjusted to pH 7.5 by the addition of N Tris base. To above solution were added inorganic pyrophosphate (10 U), fusion enzyme (5 U, PEG pellet). Again the pH was adjusted to 7.5 and the mixture was incubated for another 5 h at 37°C. The insoluble material was removed by centrifuge. The resulting solution was lyophilized and further purified by a Biogel P-2 column using water as eluent to afford 4 (26 mg, 63%) and 5 (5 mg, 11%).

For 4: Calcd for C$_{58}$H$_{94}$N$_4$O$_{41}$: 1498.4. Found: 1498.1 (ES-MS negative). Both $^1$H and $^{13}$C NMR data are summarized in Table 1.

Preparation of M$_2$C$_2$H derivative (7). To a solution of 6 (10 mg) in 0.1 M sodium acetate buffer (1 ml, pH 5.5) M$_2$C$_2$H (15 mg) in DMSO (150 μl) was added. The mixture was stirred at room temperature for 20 min, and NaCNBH$_3$ (10 mg) was then added to the mixture. The mixture was incubated at room temperature overnight. Purification by a Sephadex G-10 column, with 0.1M PBS buffer (pH 6.0) containing 5 mM EDTA, afforded 7 (9.2 mg) based on the carbohydrate content assay.

Biantennary GM3 saccharide conjugates of HSA and KLH (12 and 13). Compound 7 and thiolated HSA or KLH were mixed in PBS buffer and adjusted to pH 7.2. The mixture was incubated at room temperature for 16 h. The progress of the reaction was monitored by HPLC. Purification of the conjugates was performed on a Biogel A 0.5 column, eluted with 0.01 M PBS buffer (pH 7.3). The fractions containing conjugate were pooled, and the contents of sialic acid and protein were analyzed. HSA conjugate 12 after dialysis and lyophilize was also analyzed by MALDI-MS, and KLH conjugate 13 was subjected to quantitative sialic acid and protein contents assay. The results showed that the average molar ratios of biantennary sialyllactoside to HSA and KLH are ap-