Synthesis and Immunological Properties of N-Modified GM3 Antigens as Therapeutic Cancer Vaccines

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The problem of immunotolerance to GM3, an important tumor-associated trisaccharide antigen, seriously hinders its usage in cancer vaccine development. To solve this problem, the keyhole limpet hemocyanin (KLH) conjugates of a series of GM3 derivatives were synthesized and screened as therapeutic cancer vaccines. First, the β-linked anomeric azides of differently N-acylated GM3 analogues were prepared by a highly convergent procedure. Next, a pentenoyl group was linked to the reducing end of the carbohydrate antigens following selective reduction of the azido group. The linker was thereafter ozonolyzed to give an aldehyde functionality permitting the conjugation of the antigens to KLH via reductive amination. Finally, the immunological properties of the resultant glycoconjugates were studied in C57BL/6 mice by assessing the titers of specific antibodies induced by the GM3 analogues. While KLH−GM3 elicited low levels of immune response, the KLH conjugates of *N*-propionyl, *N*-butanoyl, *N*-*iso*-butanoyl, and *N*-phenylacetyl GM3s induced robust immune reactions with antibodies of multiple isotypes, indicating significantly improved and T-cell dependent immune responses that lead to isotype switching, affinity maturation, and the induction of immunological "memory". It was suggested that GM3PhAc−KLH is a promising vaccine candidate for glycoengineered immunotherapy of cancer with GM3 as the primary target.

Introduction

It is well-established that oncological transformations are accompanied by the change of cell surface glycosylation patterns.[1,2] The abnormal glycans expressed on tumor cells are known as tumor-associated carbohydrate antigens (TACAs). Since the early 1970s, many TACAs have been identified[2−6] and become useful molecular templates and targets in the design and development of therapeutic cancer vaccines.[4−10] Traditionally, cancer vaccines are created by coupling TACAs to a carrier protein, such as keyhole limpet hemocyanin (KLH). Resultant glycoconjugates can be employed to immunize cancer patients to fight tumors that bear the same antigens. This method has witnessed success with some TACAs, and a few cancer vaccines thus developed are now in clinical trials.[11] However, a majority of TACAs are not functional due to the problem of immunotolerance, namely, the indifference of patients' immune system to TACAs and their protein conjugates.

To overcome this problem, we have recently explored a new strategy[12] that is based upon metabolic engineering of cell surface *N*-acetylneuraminic acid (Neu5Ac), which was pioneered by Bertozzi[13−17] and Reutter.[18,19] Neu5Ac is a natural sialic acid and is significantly overexpressed by many tumors.[20] The underlining concept of our strategy is to first immunize cancer patients with a synthetic vaccine consisting of an artificial derivative or analogue of a natural TACA that has its sialic acid residues chemically modified. After an immune response specific to the artificial antigen is established, the patients are treated with correspondingly modified mannosamine, which serves as a biosynthetic precursor of modified sialic acid, to initiate the expression of the artificial antigen in place of the natural TACA on tumors. The activated immune system will thereby eliminate the marked tumor cells. The concept is further supported by recent results from Jennings' group.[21]

In principle, this strategy should be applicable to various TACAs, provided that two conditions are fulfilled. First, there must be an effective synthetic vaccine that can induce a specific immune response in cancer patients. Second, there must be a proper protocol that can be used to engineer cancer cells to express the same artificial TACA. This work is focused on the first issue, namely, the identification of an artificial antigen for vaccine design, using GM3 (Figure 1), a sialylated TACA, as the target molecule.[1,2]

GM3 is one of the most abundant TACAs on several types of tumors, such as malignant melanoma and neuroectodermal tumors.[22] Consequently, an effective vaccine based on GM3 can be widely useful. Unfortunately, GM3 is poorly immunogenic, and attempts to break its immunotolerance by traditional techniques, such as linkage to KLH covalently, only achieved limited success.[22,23] Even though the incorporation of GM3 into "very small size proteoliposomes" made of lipoproteic extracts from bacteria could improve the immunogenicity of GM3,[24] the system is complex and difficult to control.

We anticipate that our strategy might circumvent the immunotolerance problem of GM3 and result in effective cancer immunotherapy based on GM3. Therefore, we have synthesized the natural GM3 and its artificial *N*-acyl derivatives, linked them to KLH, and studied the immunological properties of the resultant glycoconjugates.

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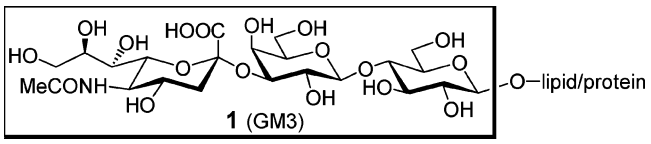
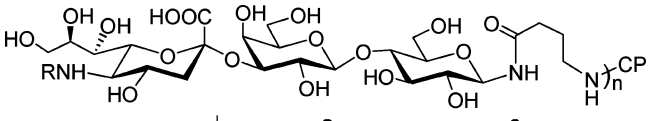


Figure 1. The structure of GM3 antigen.



R	**2** (CP = KLH)	**3** (CP = HSA)
a: Ac (natural)	CH_3CO-	CH_3CO-
b: Pr	CH_3CH_2CO-	CH_3CH_2CO-
c: Bu	$CH_3CH_2CH_2CO$-	$CH_3CH_2CH_2CO$-
d: *i*Bu	$(CH_3)_2CHCO$-	$(CH_3)_2CHCO$-
e: PhAc	$C_6H_5CH_2CO$-	$C_6H_5CH_2CO$-

Figure 2. Structures of N-modified GM3 and conjugate vaccines.

Results and Discussion

Design of Modified GM3 Antigens and Glycoconjugate Vaccines. The major objectives of this work are to understand how chemical modifications of GM3 would affect its immunogenicity and to establish effective vaccines for cancer immunotherapy using GM3. In this context, we are especially interested in modifying the sialic acid residue of GM3, because previous studies suggest that chemically modified sialic acids and sialo carbohydrates are significantly more immunogenic than their natural counterparts.[25−28] More importantly, Bertozzi,[13−17] Reutter,[18,19] and recently Varki[29] have demonstrated that a variety of cells, including tumor cells,[12,21] can uptake unnatural *N*-acyl mannosamines to biosynthesize artificial sialic acids and sialoglycoconjugates and present them onto cell surfaces. Thus, metabolic engineering of GM3 on tumor cells may be conveniently achieved by incorporating unnatural sialic acids, which will meet the second requirement of our new immunotherapy. A caveat is that in the design of modified sialo antigens we need to bear in mind that the correspondingly N-modified mannosamines have to be available to the enzymes involved in the biosynthesis of sialic acid.

We have designed and studied four unnatural *N*-acyl derivatives of GM3 (Figure 2), including *N*-propionyl GM3 (GM3NPr, **b**), *N*-butanoyl GM3 (GM3NBu, **c**), *N*-*iso*-butanoyl GM3 (GM3N*i*Bu, **d**), and *N*-phenylacetyl GM3 (GM3NPhAc, **e**), which comprise a range of structural variations. GM3NPr and natural GM3 have a small structural difference, as the propionyl group is only one carbon longer than an acetyl group. Nevertheless, a similar structural variation of polysialic acid could substantially improve the immunogenicity.[25,26] Therefore, it is interesting to examine how a propionyl group would affect the immunological properties of GM3. Butanoyl, *iso*-butanoyl, and phenylacetyl groups are structurally more different from an acetyl group, and we expect that their derivatives would be even more immunogenic. Additionally, all acyl groups in GM3NPr, GM3NBu, GM3N*i*Bu, and GM3NPhAc are more hydrophobic than the acetyl group, which we hope would further improve the immunogenicity. Our studies on sialic acid itself showed that hydrophobic substitutes could often promote immunogenicity.[30]

Our plan was to couple the *N*-acyl derivatives of GM3 to a carrier protein to form glycoconjugate vaccines, which may provide T-cell "help" for B-cell responses.[31] B-cells that express membrane-bound antibodies specific for derivatized GM3 should exhibit antibody-enhanced uptake of the glycoconjugate, allowing enhanced B-cell presentation of the carrier protein to T-cells. This mechanism drives T-cell responses that promote carbohydrate-specific B-cells to proliferate, undergo isotype class switching and affinity maturation, and differentiate into long-lived memory B-cells. Thus, conjugation of carbohydrate antigens to carrier protein enhances not only the magnitude but also the quality of antibody responses.[31]

KLH, a well-established carrier protein for experimental cancer vaccines,[6] was employed to form glycoconjugate vaccines **2a**−**e** (Figure 2), because KLH conjugates are often highly immunogenic and induce IgG antibody responses that are desirable for cancer immunotherapy. Alternatively, human serum albumin (HSA) conjugates **3a**−**e** were prepared as capture reagents for ELISA assays in immunological studies, which would avoid detection of antibodies specific for KLH.

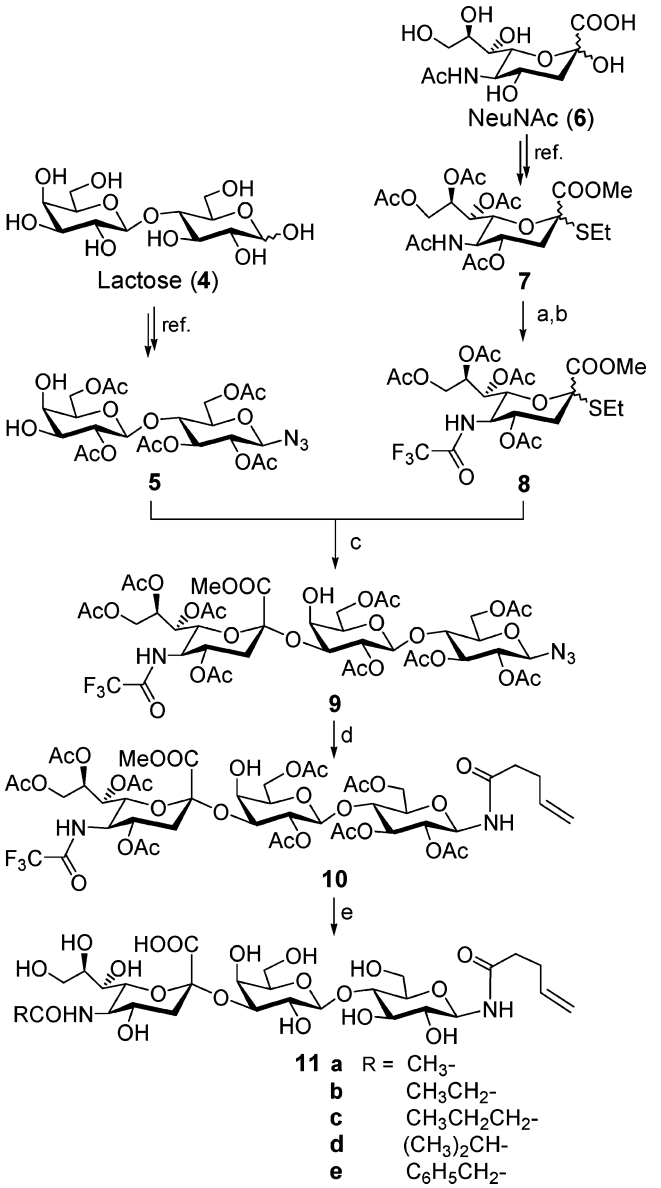
A pentenoyl group was utilized as a linker to conjugate GM3 antigens to proteins. We have recently demonstrated that as a linker a pentenoyl group has some advantages.[32] For instance, the linker and the antigens are coupled by a relatively stable amide bond through a selective reaction between pentenoyl anhydride and glycosylamines. Moreover, because the coupling reaction can be achieved at the final stage of synthesis, protection tactics required for oligosaccharide preparation can be significantly simplified. Ozonolysis of the double bond of the pentenoyl group eventually gives an aldehyde functionality that facilitates the conjugation of GM3 antigens to proteins via reductive amination.

Consequently, glycoconjugates **2a**−**e** and **3a**−**e** were synthesized for studying the immunological properties of N-modified GM3 antigens.

Synthesis of *N*-Acyl GM3 Antigens and Their Protein Conjugates. Despite the significant progress in carbohydrate chemistry in recent years, the chemical synthesis of complex oligosaccharides remains a significant undertaking. One of the major problems in the synthesis of GM3 and its derivatives is that the sialylation reactions employed to introduce the sialic acid residue usually give very poor yields and stereoselectivity.[33−39] To address this problem, we planned to examine a new strategy developed by Boons and coworkers,[40,41] in which an *N*-trifluoroacetylneuraminic acid derivative was utilized as the glycosyl donor to afford significantly improved results. The use of a trifluoroacetyl group as a protecting group for the amino group also has other advantages in our synthesis. For instance, a trifluoroacetamido group can be easily deprotected under mild conditions, which can simplify the design of protection tactics and the final stage modification of GM3.

Another important issue in the preparation of the protein conjugates of GM3 and its derivatives is that a pentenoyl group as the appropriate linker has to be

Scheme 1[a]

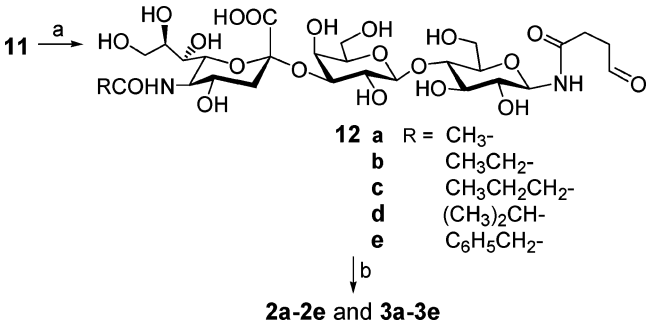


[a] Reagents and conditions: (a) MsOH (cat.), MeOH, reflux, 24 h; then CF_3COOMe, Et_3N, MeOH; (b) Ac_2O, pyridine, 84% (two steps); (c) NIS, TfOH, MS 4 Å, MeCN, ca. 63%; (d) Lindlar catalyst, H_2, MeOH/EtOAc (1:1), 4-pentenoic anhydride, 6 h, 90%; (e) 0.5 N NaOH, 10 h; then various anhydride, rt, 83−94%.

attached to the GM3 antigens. For this purpose, we planned to have the reducing end of derivatized GM3s capped by an azido group that can serve as a latent amino group. Thus, after the azido group is selectively reduced under mild conditions to give a primary amine, the pentenoyl group can be introduced to the GM3 reducing end even in the presence of free hydroxyl groups. The azido group can also serve as a protecting group, because an azido group is stable to most reactions involved in the oligosaccharide synthesis.

Meanwhile, because a trifluoroacetyl group can be selectively removed in the presence of a pentenoyl group, it will facilitate a procedure of introducing the linker at an early stage before the deprotection and acylation of the amino group on C-5. Deprotecting and modifying the sialic acid residue at the latest stage can lead to a highly convergent synthetic design as shown in Scheme 1.

Scheme 2[a]



[a] Reagents and conditions: (a) O_3, MeOH, −70 °C, 0.5 h; then Me_2S, to room temperature, 2 h, 85−90%; (b) KLH or HSA, $NaBH_3CN$, 0.1 N $NaHCO_3$, 37 °C, 3 days.

The glycosyl acceptor **5** and donor **8** were prepared from lactose (**4**) and Neu5Ac (**6**) according to reported procedures.[32,42] The azido group was attached to the glycosyl acceptor segment at an early stage. The thioglycoside of sialic acid (**7**) was smoothly converted into a trifluoroacetyl derivative **8** in two steps. The sialylation of **5** by **8** under the conventional conditions[40,41] afforded trisaccharide **9** in a good yield (ca. 63%) and excellent stereoselectivity, but the purification process was very tedious due to the partial overlapping of **9** with the excessive substrate **5** on the column. Because we found that after introduction of the linker the product was more easily purified, we directly applied the partially purified **9** to the next step. The selective reduction of the azido group of **9** and acylation of the resultant amine were performed in one pot to afford the *N*-trifluoroacetyl GM3 conjugate **10** in an excellent yield (90%). The ^1H NMR data of **10** (H-3e: δ 2.64) showed that the sialic acid residue was α-linked[33−38,40,41] and the glycosylamino bond had a β-configuration (H-1: δ 4.90, $J = 9.0$ Hz). The α-configuration of sialic acid was further confirmed in the deprotected product **11** that showed a downfield shift of H-3e NMR signal (δ 2.63) compared to that of the β-isomer (δ < 2.40). It is worth pointing out that this is the first synthesis of a glycosylazido derivative of GM3, although a number of procedures for GM3 and other derivatives have been described previously.[33−39]

After **10** was obtained, its trifluoroacetyl group was removed with 0.5 N NaOH solution at room temperature. Under this condition, the hydroxyl and carboxyl groups were also deprotected, but the linker was unaffected. The resultant free amino group was then selectively acylated in methanol by acetic, propionic, butyric, *iso*-butyric, and phenylacetic anhydrides, respectively, to afford the *N*-acyl derivatives of GM3 **11a**−**e**. The products were purified on a Biogel P2 column, and their identities and purities were characterized with MS and high-resolution NMR.

GM3 derivatives were conjugated to the carrier proteins through a two-step procedure (Scheme 2). First, each GM3 derivative was treated with ozone in methanol to selectively break the carbon−carbon double bond of the linker and give an aldehyde, as shown in **12a**−**e**. The products were purified on a Biogel P2 column, and their NMR spectra clearly showed a signal of the hydrated aldehyde at about 5.0 ppm and the disappearance of the double bond signals. The coupling of **12** to carrier proteins, including KLH and HSA, was ac-

Table 1. Carbohydrate Loading of Glycoconjugates

	KLH conjugates					HSA conjugates				
sample	**2a** (Ac)	**2b** (Pr)	**2c** (Bu)	**2d** (*i*Bu)	**2e** (PhAc)	**3a** (Ac)	**3b** (Pr)	**3c** (Bu)	**3d** (*i*Bu)	**3e** (PhAc)
loading (%)	12.4	13.5	12.8	13.5	10.8	15.0	15.4	17.5	13.2	11.9

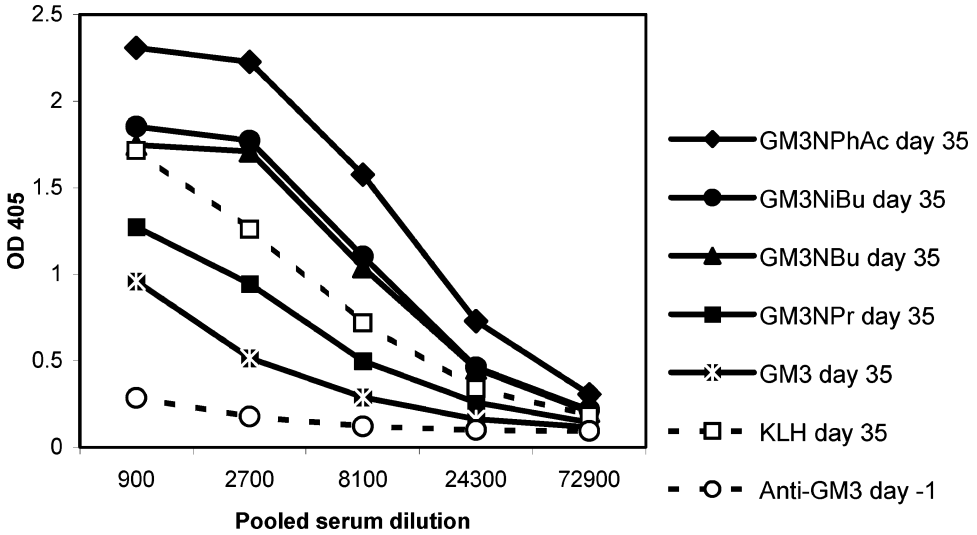


Figure 3. Antigen-specific total antibody contents in sera analyzed by ELISA. Each line represents the antibody level in serum pooled from six mice. Anti-GM3 and anti-derivatized GM3 specific antibody levels were obtained from mice immunized with various GM3−KLH glycoconjugates. Anti-KLH specific antibody level, which was used as a positive control, was obtained from mice immunized with GM3NPr, and anti-GM3 specific antibody level of preimmune serum was obtained from the same group as the negative control (equivalent results were obtained from the other groups). For ELISA assays, the corresponding GM3−HSA glycoconjugates were used as the capture antigens. Goat anti-mouse κ antibodies were used to detect antibodies bond to the capture antigens. Error bars are smaller than the symbol width.

complished by reductive amination carried out in a 0.1 M $NaHCO_3$ buffer in the presence of $NaBH_3CN$. The GM3 derivatives were employed in excess, and the glycoconjugates were readily separated from the unreacted GM3 derivatives on a Biogel A0.5 column. The product that gave positive results in assays for both sugar and protein was naturally the first component eluted out of the column and was collected, dialyzed against distilled water, and finally freeze-dried to afford the glycoconjugates as white fluffy solids.

The sialic acid contents of the resultant glycoconjugates were analyzed by the Svennerholm[43] method and then converted into the levels of carbohydrate loading according to the equation presented in the Experimental Section. In general, the coupling reactions were very efficient and gave glycoconjugates containing 11−18% (w/w) of carbohydrates (Table 1). These levels of antigen loading are at the ideal range for glycoconjugates being used as vaccines in immunological studies[26] and as capture reagents in ELISA assays.

Immunological Studies of Protein Conjugates of *N*-Acyl GM3 Antigens. The immunogenicity of the KLH conjugates of *N*-acyl GM3 derivatives was investigated in C57BL/6 mice, a well-characterized inbred strain that is appropriate for further studies. As mentioned earlier, conjugating GM3 derivatives to a carrier protein, namely, KLH herein, allows B-cells to present GM3 derivative specific immunogenic peptides, generated by proteolytic cleavage of the KLH molecule, to T-cells, which should lead to a significantly improved immune response.[31]

The immunizations made use of Ribi adjuvant, an oil emulsion system that is approved for use in humans.[44] At day 27 and day 35 following intraperitoneal immunizations (Experimental Section), animal sera were withdrawn and pooled with respect to the individual group and date. We assessed the total antigen-specific antibody titers of the pooled sera by means of ELISA. Then, the titers of specific antibody isotypes, including IgM, IgG1, IgG2a, and IgG3, were assessed. The isotypes of the antibodies have different capacities for inducing the effector function important to antitumor response (e.g., antibody-dependent cell-mediated cytotoxicity and the ability to fix complement on antibody bound membranes).[6,45,46] The sera obtained at day 27 and day 35 gave similar results in terms of different GM3 antigens, but only the results of day 35 are shown and discussed here.

Figure 3 shows the total antibody titers with the readings of optical density (OD) plotted against pooled sera dilutions in an ELISA assay in which plate wells were coated with a derivatized GM3−HSA conjugate or KLH protein alone as the capture antigen. Higher OD values indicate higher concentrations of serum antibodies specific for the capture antigen. These wells were bound with half-log dilutions of pooled sera from day −1 and day 35 bleeds of six immunized mice per derivatized GM3−KLH conjugate. Pooling the serum of the six immunized mice gives a mean serum antibody concentration of the six immunized mice. Serum antibodies bound to the capture antigen were detected with goat anti-mouse κ light chain specific and horseradish peroxidase (HRP) conjugated antibody. In mice, κ light chain antibodies constitute about 95% of all antibodies, so this assay provides a good approximation of total antibody response.[46] Using the GM3−HSA conjugates

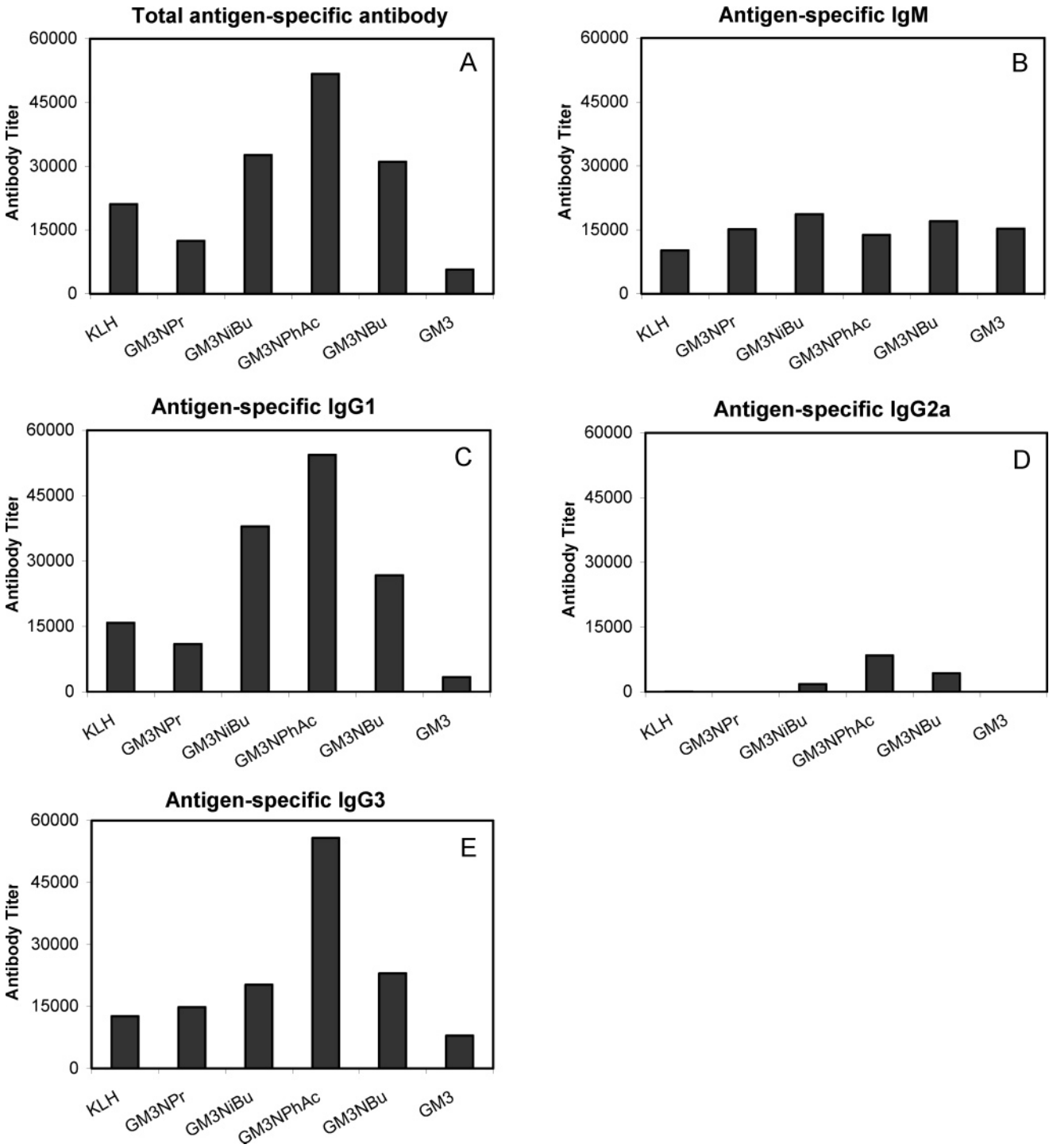


Figure 4. Titer analysis of antigen-specific antibodies determined by ELISA assay (Experimental Section). Each represents the titer in pooled serum obtained on day 35 after primary and booster immunizations (Experimental Section) from six replicate animals.

as capture antigens allows us to detect those antibodies that are specific for the modified GM3 component of the conjugates because the mice have never been exposed to HSA and the serum therefore will not contain antibodies to this protein. In fact, none of the pooled antisera showed binding to HSA protein (data not shown). However, the experiment using KLH as the capture antigen allows the determination of a positive control, because KLH is well-established as a strong immunogen and will elicit a high titer of antibodies specific for KLH. Figure 3 indicates that although all of the tested glycoconjugates (**2a**−**e**) were immunogenic, GM3NPhAc showed the highest level of antigen-specific antibodies. The natural GM3 antigen gave the lowest concentration of GM3-specific antibodies. There was essentially no binding of preimmune serum to various GM3−HSA conjugates.

Although the raw data allows us to roughly estimate the relative immunogenicities, a titer analysis was performed to normalize the data and quantify more accurately the immunogenicities of the derivatized glycoconjugates (Figure 4). Panel A of Figure 4 shows titers of total glycoconjugate-specific antibodies, and as above, the assays measured the κ light chain. A hier-

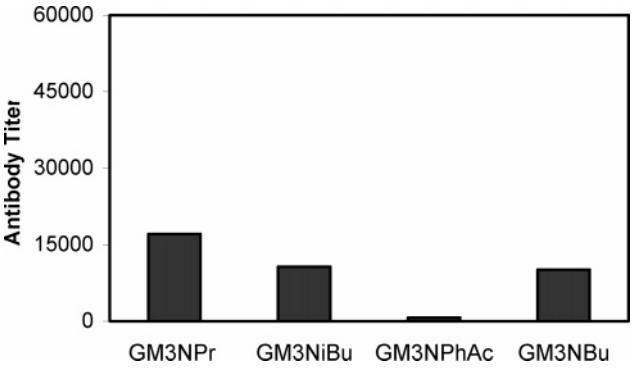


Figure 5. Titer analysis of antibodies cross reactive with the natural GM3 determined by ELISA (Experimental Section). ELISA plates were coated with GM3−HSA conjugate. Each bar represents total anti-GM3 reactivity in pooled sera from six replicate animals immunized with the indicated derivatized GM3.

archy of immunogenicity was evident. GM3NPhAc was the most immunogenic, followed by GM3NiBu, GM3NBu, and then GM3NPr. The natural GM3 antigen induced the lowest concentration of serum antibodies.

Panels B−E of Figure 4 show the titers of different antibody isotypes. It was observed that different conjugates produced relatively equivalent concentrations of IgM antibody (panel B). IgM antibody has important complement fixing functions. However, because it is the first antibody produced by antigen-specific B-cells in a T-cell independent manner and it is not subject to affinity maturation, IgM immune responses are limited by lack of memory.[31] Major titer differences were observed with IgG responses to derivitized GM3 antigens. The IgG1 antibody titers seen in panel C of Figure 4 were substantially higher than that of IgM, and the glycoconjugates displayed the same hierarchy of immunogenicity seen in panel A (i.e., GM3NPhAc being the most immunogenic, followed by GM3NiBu, GM3NBu, and GM3NPr). The natural GM3 induced very low levels of IgG1 antibody. Panel D of Figure 4 shows that all of the tested glycoconjugates gave very low IgG2a antibody levels with only GM3PhAc, GM3NiBu, and GM3NBu having measurable IgG2a titers. IgG3 titers (Figure 4, panel E) showed the same hierarchy as seen in panels A and C with a more pronounced difference in the magnitude of the GM3NPhAc titer relative to the other gycoconjugates.

We next examined the reactivity of sera from mice immunized with derivatized GM3 conjugates to the HSA conjugate of natural GM3 (Figure 5). This allowed us to assess the potential for autoimmune reactions in response to immunization with the derivatized glycoconjugates. In general, sera from these animals showed much lower reactivity with GM3−HSA than that with the correspondingly modified GM3−HSA shown in Figure 4A. This was especially true for GM3NPhAc. In fact, the cross reactivity of GM3NPhAc sera to the natural GM3 was almost negligible. Moreover, the hierarchy of cross-reactivity titers is the inverse of the titers of immunogenicity, with GM3NPhAc sera the least cross-reactive, followed by GM3NBu and GM3NiBu. However, the reactivity of GM3NPr sera to GM3NPr and the natural GM3 were rather similar, suggesting that the immune response induced by GM3NPr had little selectivity. The result of GM3NPhAc shown in Figure 5 can also help to conclude that the linker could not induce significant antibody responses or a strong cross reactivity should be observed even for GM3NPhAc. These results are understandable. At the molecular level, GM3PhAc is the most dissimilar to the natural GM3. Therefore, GM3PhAc should be the most immunogenic, while antibodies against this structure should be least likely to bind to the natural form of GM3. However, GM3NPr is very similar to the natural GM3, so it was less immunogenic, and its antibodies had the highest level of cross reactivity with the natural GM3.

In summary, an expedient method was established to synthesize N-modified derivatives of GM3 and their protein conjugates. The properties of these glycoconjugates were studied to explore the impact of structural changes of GM3 on its immunogenicity. It was revealed that derivatized GM3 antigens are generally more immunogenic than GM3 itself. Moreover, whereas GM3 induces mainly IgM responses, derivatized GM3 antigens induce both IgM and IgG responses. From the viewpoint of cancer therapy, IgG responses are more interesting because of their desirable properties such as immunological memory, affinity maturation, and improved antibody-dependent cell-mediated cytotoxicity.[45,46]

GM3NPhAc proved to be the most immunogenic among the GM3 derivatives investigated, and its antisera were the least cross reactive with the natural GM3. These results indicate that GM3NPhAc is an excellent candidate as an antitumor vaccine. Meanwhile, our studies[47] have also shown that the *N*-phenylacetyl derivative of mannoseamine is one of the best substrates for *N*-acetyl sialic acid adolase, an enzyme that may be involved in bypassing the biosynthetic bottleneck in the glycoengineering of sialic acid and sialoglycoconjugates on cells.[48] In view of these results, we anticipate that the combination of a GM3PhAc-based vaccine with the glycoengineering of cancer cells by *N*-phenylacetylmannosamine holds great promises as an effective immunotherapy of cancer. In pursuit of this goal, we are presently focusing on the in vivo studies of the treatment of melanoma by using *N*-phenylacetylmannoseamine as the glycoengioneering precursor and GM3PhAc conjugates as the vaccines.

Experimental Section

***N*-{*O*-[Methyl 4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-5-trifluoroacetamido-D-glycero-α-D-galacto-non-2-ulopyranosylonate]-(2→3)-*O*-(2,6-di-*O*-acetyl-*β*-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-*β*-D-glucopyranosyl} 4-Pentenamide (10).** After a mixture of **8** (500 mg, 0.85 mmol), **5** (982 mg, 1.7 mmol), and activated molecular sieves (4 Å, 2.0 g) in anhydrous acetonitrile (5.0 mL) was stirred at room temperature for 20 h under N_2, the mixture was cooled to −35 °C, and NIS (1.7 mmol) and TfOH (0.2 mmol) were added. It was kept at −35 °C for 1 h and then diluted with DCM (20 mL). The solid material was filtered off and washed with DCM (5 mL). The combined filtrates were washed with aqueous $Na_2S_2O_3$ (20%) and water. The organic phase was dried over Na_2SO_4 and concentrated under reduced pressure. Silica gel column chromatography of the residue afforded the desired trisaccharide **9** (585 mg, 0.53 mmol, 63%) containing a small amount of impurities. A solution of **9** (550 mg, 0.5 mmol) and 4-pentenoic anhydride (20 mg) in MeOH and EtOAc (1:1, v/v, 5 mL) was stirred vigorously with Lindlar catalyst (60 mg) and H_2 at room temperature for 3 h. The solid material was filtered off, and the filtrate was condensed under reduced

pressure. The residue was purified by column chromatography to afford **10** (515 mg, 90%) as a white solid: R_f 0.30 (acetone and toluene 1:1); $[\alpha]_D$ +2.4 (*c* 1.0, $CHCl_3$). ^1H NMR ($CDCl_3$, 600 MHz): δ 7.13 (d, 1 H, *J* 7.8 Hz, NH), 6.25 (d, 1 H, *J* 9.2 Hz, NH), 5.70 (m, 1 H, *J* 6.0, 10.8, 16.8 Hz, CH_2=C*H*), 5.39 (m, 1 H, *J* 4.2 Hz, H-8″), 5.34 (d, 1 H, *J* 8.4 Hz, H-7″), 5.19 (dd, 1H, *J* 9.0, 10.2 Hz, H-2), 5.18 (t, 1 H, *J* 10.2 Hz, H-3), 4.98 (d, 1 H, *J* 16.8 Hz, C*H*$_2$=CH), 4.92 (d, 1 H, *J* 10.2 Hz, C*H*$_2$=CH), 4.90 (d, 1 H, *J* 9.0 Hz, H-1), 4.88 (dd, 1 H, *J* 5.0, 10.8 Hz, H-4″), 4.76 (dd, 1H, *J* 9.0, 9.6 Hz, H-2′), 4.46 (d, 1 H, *J* 7.8 Hz, H-1′), 4.38 (d, 1 H, *J* 11.4 Hz, H-9″a), 4.33 (d, 1 H, *J* 10.8 Hz, H-6″), 4.22 (d, 1 H, *J* 10.2 Hz, H-3′), 4.7 (m, 2 H), 4.13 (dd, 1 H, *J* 12.0, 4.2 Hz, H-6), 4.04−4.00 (m, 2 H), 3.94 (q, 1 H, *J* 10.2 Hz, H-5″), 3.74 (m, 1 H, *J* 9.0 Hz, H-4), 3.72 (s, 3 H), 3.67 (d, 1 H, *J* 8.4 Hz, H-5), 3.58 (t, 1 H, *J* 6.0 Hz, H-5′), 3.35 (bs, 1 H, H-4′), 2.82 (d, 1 H, *J* 3.6 Hz, OH), 2.64 (m, H-3″e), 2.26 (m, 2 H), 2.18 (m, 2 H), 2.06, 2.03, 2.02, 2.01, 2.00, 1.98, 1.97, 1.96, 1.94 (9s, 9 × 3H, OAc), 1.70 (dd, 1 H, *J* 12.6, 12.0 Hz, H-3″a). ^{19}F NMR ($CDCl_3$): δ −76.7 (s). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 172.7, 171.2, 170.9, 170.7, 170.6, 170.6, 170.5, 169.8, 169.7, 169.4, 168.2, 158.0 (q, *J* 37.6 Hz), 136.4, 115.8, 115.0 (q, *J* 285 Hz), 100.8, 96.9, 77.9, 75.1, 74.5, 72.5, 71.7, 71.0, 69.6, 68.6, 68.5, 68.4, 66.9, 66.8, 62.7, 62.1, 61.9, 53.2, 48.7, 37.8, 35.6, 28.9, 21.4, 20.9, 20.8, 20.7, 20.7, 20.6, 20.5, 20.4, 20.3. FABMS Calcd for $C_{47}H_{64}F_3N_2O_{28}$ $[M + H]^+$: 1161.4. Found: 1162.0. Calcd for $C_{47}H_{63}F_3N_2NaO_{28}$ $[M + Na]^+$: 1183.4. Found: 1184.0.

***N*-{*O*-[5-Acylamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid]-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranosyl} 4-Pentenamide (11a−e).** Compound **10** (500 mg, 0.45 mmol) was dissolved in 0.5 N NaOH (30 mL), and the mixture was stirred at room temperature for 10 h. After neutralization and condensation under reduced pressure, the crude product was directly used for the acylation. To a solution of the resultant amine (20 mg, 0.03 mmol) in 2.5 mL of MeOH and 0.5 mL of NaOH (0.5 N) was added 0.1 mL of an acyl anhydride dropwise in an ice−water bath. After the reaction finished within 6 h as indicated by TLC, the mixture was condensed under reduced pressure. The residue was purified on a Biogel P-2 column with H_2O as the eluent. Fractions containing the expected product were combined and freeze-dried to afford various *N*-acyl GM3 derivatives **11a**−**e**.

***N*-{*O*-[5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid]-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranosyl} 4-Pentenamide (11a**, 20 mg, 92%): $[\alpha]_D$ 2.4 (*c* 0.7, MeOH). ^1H NMR (D_2O, 600 MHz): δ 5.73 (m, 1 H, *J* 6.6, 10.2, 17.2 Hz, CH_2=C*H*), 4.96 (dd, 1 H, *J* 1.8, 17.2 Hz, C*H*$_2$=CH), 4.90 (dd, 1 H, *J* 1.8, 10.2 Hz, C*H*$_2$=CH), 4.83 (d, 1 H, *J* 9.0 Hz, H-1), 4.39 (d, 1 H, *J* 7.8 Hz, H-1′), 3.96 (dd, 1 H, *J* 3.0, 9.6 Hz), 3.80 (dd, 1 H, *J* 1.8, 12.6 Hz), 3.66−3.45 (dd, 1 H, *J* 1.8, 10.2 Hz), 3.44 (dd, 1 H, *J* 9.6, 10.2 Hz), 3.28 (dd, 1 H, *J* 9.0, 9.6 Hz), 2.61 (dd, 1 H, *J* 12.6, 4.8 Hz, H-3″e), 2.28 (m, 2 H), 2.24 (m, 2 H), 1.88 (s, 3 H, $COCH_3$), 1.65 (t, 1 H, *J* 12.0 Hz, H-3″a). FABMS Calcd for $C_{28}H_{46}N_2NaO_{19}$ $[M + Na]^+$: 737.2. Found: 737.3. Calcd for $C_{28}H_{45}N_2Na_2O_{19}$ $[M - H + 2Na]^+$: 759.2. Found: 759.3. Calcd for $C_{32}H_{58}N_3O_{21}$ $[M + DEA + H]^+$: 820.3. Found: 820.4. HR-FABMS Calcd for $C_{28}H_{46}N_2NaO_{19}$ $[M + Na]^+$: 737.2592. Found: 737.2422.

***N*-{*O*-[5-Propionylamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid]-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranosyl} 4-Pentenamide (11b**, 20 mg, 91%): $[\alpha]_D$ 2.1 (*c* 1.0, MeOH). ^1H NMR (D_2O, 600 MHz): δ 5.74 (m, 1 H, *J* 6.6, 10.2, 16.8 Hz, CH_2=C*H*), 4.96 (dd, 1 H, *J* 1.8, 17.4 Hz, C*H*$_2$=CH), 4.91 (dd, 1 H, *J* 1.8, 9.6 Hz, C*H*$_2$=CH), 4.83 (d, 1 H, *J* 9.0 Hz, H-1), 4.39 (d, 1 H, *J* 8.4 Hz, H-1′), 3.80 (dd, 1 H, *J* 2.4, 12.6 Hz), 3.42 (t, 1 H, *J* 10.2 Hz), 3.28 (dd, 1 H, *J* 9.0, 9.6 Hz), 2.62 (dd, 1 H, *J* 12.6, 4.8 Hz, H-3″e), 2.28 (m, 2 H), 2.24 (m, 2 H), 2.15 (q, 2 H, *J* 7.8 Hz, CH_3CH_2CO), 1.79 (t, 1 H, *J* 12.6 Hz, H-3″a), 0.91 (t, 3 H, *J* 7.8 Hz, CH_3CH_2CO). FABMS Calcd for $C_{29}H_{48}N_2NaO_{19}$ $[M + Na]^+$: 751.3. Found: 751.4. Calcd for $C_{29}H_{47}N_2Na_2O_{19}$ $[M - H + 2Na]^+$: 773.3. Found: 773.5. Calcd for $C_{33}H_{60}N_3O_{21}$ $[M + DEA + H]^+$: 834.4. Found: 834.7. HR-FABMS Calcd for $C_{29}H_{48}N_2NaO_{19}$ $[M + Na]^+$: 751.2749. Found: 751.2532.

***N*-{*O*-[5-Butanoylamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid]-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranosyl} 4-Pentenamide (11c**, 19 mg, 87%): $[\alpha]_D$ 3.1 (*c* 0.7, MeOH). ^1H NMR (D_2O, 600 MHz): δ 5.74 (m, 1 H, *J* 6.6, 10.2, 16.8 Hz, CH_2=C*H*), 4.96 (dd, 1 H, *J* 1.2, 17.4 Hz, C*H*$_2$=CH), 4.90 (d, 1 H, *J* 10.2 Hz, C*H*$_2$=CH), 4.83 (d, 1 H, *J* 9.6 Hz, H-1), 4.39 (d, 1 H, *J* 7.8 Hz, H-1′), 3.97 (dd, 1 H, *J* 3.0, 9.6 Hz), 3.42 (dd, 1 H, *J* 1.8, 9.0 Hz), 3.28 (dd, 1 H, *J* 9.0, 9.6 Hz), 2.62 (dd, 1 H, *J* 12.0, 4.2 Hz, H-3″e), 2.29 (m, 2 H), 2.24 (m, 2 H), 2.13 (t, 2 H, *J* 7.2 Hz, $COCH_2CH_2CH_3$), 1.66 (t, 1 H, *J* 12.0 Hz, H-3″a), 1.47 (m, 2 H, $COCH_2CH_2CH_3$), 0.77 (t, 3 H, J 7.8 Hz, $COCH_2CH_2CH_3$). FABMS Calcd for $C_{30}H_{50}N_2NaO_{19}$ $[M + Na]^+$: 765.3. Found: 765.4. Calcd for $C_{30}H_{49}N_2Na_2O_{19}$ $[M - H + 2Na]^+$: 787.3. Found: 787.3. Calcd for $C_{34}H_{62}N_3O_{21}$ $[M + DEA + H]^+$: 848.4. Found: 848.8. HR-FABMS Calcd for $C_{34}H_{62}N_3O_{21}$ $[M + DEA + H]^+$: 848.3876. Found: 848.3874.

***N*-{*O*-[5-*iso*-Butanoylamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid]-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranosyl} 4-Pentenamide (11d**, 21 mg, 94%): $[\alpha]_D$ 2.8 (*c* 1.0, MeOH). ^1H NMR (D_2O, 600 MHz): δ 5.74 (m, 1 H, *J* 6.0, 10.2, 16.8 Hz, CH_2=C*H*), 4.95 (d, 1 H, *J* 16.2 Hz, C*H*$_2$=CH), 4.90 (d, 1 H, *J* 10.2 Hz, C*H*$_2$=CH), 4.83 (d, 1 H, *J* 9.0 Hz, H-1), 4.39 (d, 1 H, *J* 7.2 Hz, H-1′), 3.97 (dd, 1 H, *J* 3.0, 9.6 Hz), 3.28 (t, 1 H, *J* 9.0 Hz), 2.62 (dd, 1 H, *J* 12.0, 4.2 Hz, H-3″e), 2.38 (m, 1 H, COC*H*Me$_2$), 2.29 (m, 2 H), 2.20 (m, 2 H), 1.66 (t, 1 H, *J* 12.0 Hz, H-3″a), 0.96, 0.95 (2 d, 2 × 3 H, J 6.6 Hz, $COCHMe_2$). HR-FABMS Calcd for $C_{30}H_{50}N_2NaO_{19}$ $[M + Na]^+$: 765.2905. Found: 765.2936. Calcd for $C_{30}H_{49}N_2Na_2O_{19}$ $[M - H + 2Na]^+$: 787.2725. Found: 787.2728.

***N*-{*O*-[5-Phenylacetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic Acid]-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranosyl} 4-Pentenamide (11e**, 20 mg, 83%): $[\alpha]_D$ 1.1 (*c* 0.7, MeOH). ^1H NMR (D_2O, 600 MHz): δ 7.25−7.15 (m, 5 H), 5.73 (m, 1 H, *J* 7.0, 10.2, 16.8 Hz, CH_2=C*H*), 4.96 (d, 1 H, *J* 1.2, 17.4 Hz, C*H*$_2$=CH), 4.89 (d, 1 H, *J* 10.2 Hz, C*H*$_2$=CH), 4.83 (d, 1 H, *J* 9.0 Hz, H-1), 4.37 (d, 1 H, *J* 7.8 Hz, H-1′), 3.99 (dd, 1 H, *J* 2.0, 9.6 Hz), 3.42 (s, 2 H, PhC*H*$_2$), 3.28 (t, 1 H, *J* 7.2 Hz), 3.22 (dd, 1 H, *J* 9.0 Hz), 2.62 (dd, 1 H, *J* 4.0, 12.2 Hz, H-3″e), 2.29 (m, 2 H), 2.21 (m, 2 H), 1.77 (t, 1 H, *J* 12.2 Hz, H-3″a). HR-FABMS Calcd for $C_{34}H_{50}N_2NaO_{19}$ $[M + Na]^+$: 813.2905. Found: 813.2907. Calcd for $C_{34}H_{49}N_2Na_2O_{19}$ $[M - H + 2Na]^+$: 835.2725. Found: 835.2750.

***N*-{*O*-[5-Acylamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid]-(2→3)-*O*−(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranosyl} 4-Oxo-butanamide (12a−e)**. Ozone was bubbled into individual solutions of **11a**−**e** (0.02 mmol) in MeOH (5 mL) at −78 °C until a blue color appeared and remained (ca. 30 min). The solutions were kept at −78 °C for another 10 min, and then nitrogen was introduced to remove the remaining ozone. After Me_2S (0.2 mL) was added at −78 °C, the resulting solution was allowed to warm to room temperature over a period of 1 h and stand for another 1 h before it was condensed in a vacuum. The crude product was purified on a Sephadex G10 column using distilled water as the eluent to give the aldehyde products **12a**−**e** as white solids, which were directly used in the conjugation reactions without further purification.

***N*-{*O*-[5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid]-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranosyl} 4-Oxo-butanamide (12a)**. ^1H NMR (D_2O, 300 MHz): δ 5.05 (d, 1 H, *J* 9.0 Hz, H-1), 4.98 (t, 1 H, *J* 6.0 Hz, CHO), 4.54 (d, 1 H, *J* 7.7 Hz, H-1′), 3.96 (dd, 1 H, *J* 2.7, 9.7 Hz), 2.76 (dd, 1 H, *J* 12.6, 4.9 Hz, H-3″e), 2.46 (m, 1 H), 2.38 (m, 2 H), 2.02 (s, 3 H, $COCH_3$), 1.9 (m, 1 H), 1.79 (t, 1 H, *J* 12.5 Hz, H-3″a). FABMS Calcd for $C_{27}H_{44}N_2NaO_{20}$ $[M + Na]^+$: 739.3. Found 739.4. Calcd for $C_{27}H_{44}N_2KO_{20}$ $[M + K]^+$: 755.2. Found 755.4. HR-FABMS Calcd for $C_{31}H_{53}N_3NaO_{21}$ $[M - H_2O + DEA + Na]^+$: 826.3067. Found:

826.3034. Calcd for $C_{31}H_{54}N_3O_{21}$ [M − H_2O + DEA + H]$^+$: 804.3247. Found: 804.3247.

***N*-{*O*-[5-Propionylamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid]-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranosyl} 4-Oxo-butanamide (12b)**. ^{1}H NMR (D_2O, 600 MHz): δ 4.88 (d, 1 H, *J* 9.0 Hz, H-1), 4.72 (t, 1 H, *J* 6.0 Hz, CHO), 4.40 (d, 1 H, *J* 7.8 Hz, H-1′), 3.98 (dd, 1 H, *J* 2.4, 9.7 Hz), 2.62 (dd, 1 H, *J* 12.6, 4.8 Hz, H-3″e), 2.50 (m, 1 H), 2.29 (m, 2 H), 2.16 (q, 2 H, *J* 7.8 Hz, COCH_2CH$_3$), 1.80 (m, 1 H), 1.67 (t, 1 H, *J* 12.0 Hz, H-3″a), 0.97 (t, 3 H, *J* 7.2 Hz, COCH$_2$CH_3). FABMS Calcd for $C_{28}H_{46}N_2NaO_{20}$ [M + Na]$^+$: 753.3. Found: 753.4. Calcd for $C_{28}H_{46}N_2KO_{20}$ [M + K]$^+$: 769.3. Found: 769.4. HR-FABMS Calcd for $C_{32}H_{55}N_3NaO_{21}$ [M − H_2O + DEA + Na]$^+$: 840.3225. Found: 840.3286. Calcd for $C_{32}H_{56}N_3O_{21}$ [M − H_2O + DEA + H]$^+$: 818.3404. Found 818.3405.

***N*-{*O*-[5-Butanoylamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid]-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranosyl} 4-Oxo-butanamide (12c)**. ^{1}H NMR (D_2O, 600 MHz): δ 4.85 (d, 1 H, *J* 9.0 Hz, H-1), 4.70 (t, 1 H, *J* 4.8 Hz, CHO), 4.40 (d, 1 H, *J* 7.8 Hz, H-1′), 3.96 (dd, 1 H, *J* 2.7, 9.7 Hz), 2.60 (dd, 1 H, *J* 12.6, 4.8 Hz, H-3″e), 2.40 (m, 1 H), 2.28 (m, 2 H), 2.11 (t, 2 H, *J* 7.2 Hz, COCH_2), 1.83 (m, 1 H), 1.65 (t, 1 H, *J* 12.0 Hz, H-3″a), 1.47 (m, 2 H, COCH$_2$CH_2), 0.75 (t, 3 H, *J* 7.2 Hz, COCH$_2$CH$_2$CH_3). FABMS Calcd for $C_{29}H_{47}N_2Na_2O_{20}$ [M − H + 2Na]$^+$: 789.3. Found 789.5. HR-FABMS Calcd for $C_{33}H_{57}N_3NaO_{21}$ [M − H_2O + DEA + Na]$^+$: 854.3380. Found: 854.3334. Calcd for $C_{33}H_{58}N_3O_{21}$ [M − H_2O + DEA + H]$^+$: 832.3560. Found: 832.3556.

***N*-{*O*-[5-*iso*-Butanoylamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid]-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranosyl} 4-Oxo-butanamide (12d)**. ^{1}H NMR (D_2O, 600 MHz): 4.90 (d, 1 H, *J* 9.0 Hz, H-1), 4.82 (t, 1 H, *J* 6.0 Hz, CHO), 4.40 (d, 1 H, *J* 7.8 Hz, H-1′), 3.85 (t, 1 H, *J* 9.6 Hz), 3.44 (dd, 1 H, *J* 8.0, 10.0 Hz), 3.40 (dd, 1 H, *J* 1.8, 9.6 Hz), 2.62 (dd, 1 H, *J* 12.6, 4.8 Hz, H-3″e), 2.57 (m, 1 H), 2.38 (m, 1 H, COC*H*), 2.32 (m, 1 H), 2.25 (m, 1 H), 1.82 (m, 1 H), 1.66 (t, 1 H, *J* 12.0 Hz, H-3″a), 0.96, 0.95 (2d, 6 H, *J* 6.6 Hz, CH*Me*$_2$). FABMS Calcd for $C_{29}H_{48}N_2NaO_{20}$ [M + Na]$^+$: 767.3. Found: 767.3. Calcd for $C_{29}H_{48}N_2KO_{20}$ [M + K]$^+$: 783.3. Found: 783.3. HR-FABMS Calcd for $C_{33}H_{57}N_3NaO_{21}$ [M − H_2O + DEA + Na]$^+$: 854.3380. Found: 854.3367. Calcd for $C_{33}H_{58}N_3O_{21}$ [M − H_2O + DEA + H]$^+$: 832.3560. Found: 832.3576.

***N*-{*O*-[5-Phenylacetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid]-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranosyl} 4-Oxo-butanamide (12e)**. ^{1}H NMR (D_2O, 600 MHz): δ 7.25−7.18 (m, 5 H), 4.90 (d, 1 H, *J* 9.6 Hz, H-1), 4.41 (t, 1 H, *J* 7.2 Hz, CHO), 4.39 (d, 1 H, *J* 7.8 Hz, H-1′), 3.95 (dd, 1 H, *J* 3.0, 9.6 Hz), 3.84 (t, 1 H, *J* 9.6 Hz), 3.48 (s, 2 H, PhCH_2), 3.43 (dd, 1 H, *J* 9.6, 7.8 Hz), 3.22 (d, 1 H, *J* 9.0 Hz), 2.62 (dd, 1 H, *J* 4.8, 12.6 Hz, H-3″e), 2.55 (m, 1 H), 2.23−2.33 (m, 2 H), 1.82 (m, 1 H), 1.65 (t, 1 H, *J* 12.0 Hz, H-3″a). FABMS Calcd for $C_{33}H_{48}N_2NaO_{20}$ [M + Na]$^+$: 815.2698. Found: 815.3. Calcd for $C_{33}H_{48}N_2KO_{20}$ [M + K]$^+$: 831.3. Found: 831.3. HR-FABMS Calcd for $C_{37}H_{57}N_3NaO_{21}$ [M − H_2O + DEA + Na]$^+$: 902.3380. Found: 902.3360. Calcd for $C_{37}H_{58}N_3O_{21}$ [M − H_2O + DEA + H]$^+$: 880.3560. Found: 880.3510.

General Procedure for the Coupling between 12a−e and KLH or HSA. A solution of **12a−e** (6 mg each), KLH or HSA (5 mg), and $NaBH_3CN$ (5 mg) in 0.1 M $NaHCO_3$ (0.4 mL, pH 7.5−8.0) was allowed to stand at room temperature in the dark for 3 days with occasional shaking. The reaction mixture was then loaded onto a Biogel A0.5 column (1 cm × 15 cm) and eluted with 0.1 M PBS buffer (*I* = 0.1, pH = 7.8). The fractions containing the glycoconjugate, characterized by BCA assay for proteins and by the Svennerholm [43] method for sialic acid, were combined and dialyzed against distilled water for 2 days. It was then lyophilized to give a white powder of the expected glycoconjugate.

Analysis of the Carbohydrate Loading Levels of the Glycoconjugates. After the solution of an exactly weighed sample of the glycoconjugate (0.5 mg) in distilled water (1.0 mL) was mixed well with the resorcinol reagent (2.0 mL), the mixture was heated in a boiling water bath for 30 min. It was then cooled to room temperature, and to the mixture was added an extraction solution (1-butanol acetate and 1-butanol, 85:15 v/v, 3.0 mL). The mixture was shaken vigorously before it was allowed to stand still for ca. 10 min, allowing the organic layer to separate well from the inorganic layer. The organic layer was transferred to a 1.0 cm cuvette, and its absorbance at 580 nm was determined by an UV−vis spectrometer, using a blank organic solution as the control. The sialic acid content of the glycoconjugate is determined against a calibration curve created with the standard NeuNAcyl (Acyl = Ac, Pr, Bu, ^{i}Bu, PhAc) solutions analyzed under the same conditions.[43] The carbohydrate loading of each glycoconjugate was calculated according to the equation shown below.

$$N\text{-Acyl GM3 loading (\%)} = \frac{\text{amount of sialic acid (mg) in the sample}}{\text{weight of the glycoconjugate sample (mg)}} \times \frac{\text{molecular weight of GM3}}{\text{molecular weight of sialic acid}} \times 100\%$$

Immunization of Mice. Six female C57BL/6 mice at the age of 8 weeks (Jackson Laboratories, Bar Harbor, ME) were immunized for each GM3−KLH glycoconjugate. Immunizations were intraperitoneal with glycoconjugate containing 2 μg of carbohydrate in 200 μL of saline mixed with 200 μL of MPL/TDM Ribi adjuvant (Sigma Chemical, St. Louis, MO) following the manufacturer's protocol. The mice were boosted with identical immunizations on days 14, 21, and 28 following the initial immunization. The mice were bled by tail vein prior to the initial immunization on day 0 and after immunization on day 27 and day 35. Bleed was clotted to obtain sera, which were stored at −80 °C.

Protocols for ELISA Analysis. ELISA plates were coated with KLH or GM3 and GM3 derivatives conjugated to HSA. These capture reagents allowed detection of antibodies specific for KLH or the GM3 and derivatized GM3 components of the glycoconjugates. Maxisorp ELISA plates (NuncNalgene, Rochester, NY) were coated overnight at 4 °C with 100 μL of HSA glycoconjugates or KLH (1 μg/mL 0.1 M bicarbonate buffer), and then washed with PBS. Sera from the six mice per group were pooled, diluted 1:300 to 1:72 900 in serial half-log dilutions in PBS with 0.02% azide, and incubated overnight in the coated ELISA plates (100 μL/well). The plates were then washed and incubated with 1:1000 dilution of alkaline phosphatase linked anti-κ, anti-IgM, or anti-IgG2a antibodies or with 1:2000 dilution of anti-IgG1 or anti-IgG3 antibodies (Southern Biotechnology, Buckingham, AL) for 1 h at room temperature. Plates were washed and developed with PNPP substrate for colorimetric readout using a BioRad 550 plate reader (BioRad, Hercules, CA) at 405 nM wavelength.

A titer analysis was performed to normalize data and calculate relative immunogenicities of derivatized glycoconjugates.[30] Optical density (OD) values were plotted against dilution values, and a best-fit line was obtained. The equation of this line was used to calculate the dilution value at which an OD of 0.5 was achieved, and antibody titer was calculated at the inverse of this dilution value.

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Supporting Information Available: Experimental procedures for **5** and **8**, and the ^{1}H NMR and MS spectra of compounds **10** and **11a−e**. This material is available free of charge via the Internet at http://pubs.acs.org.

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