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Immunogenicity Study of Globo H Analogues with Modification at the Reducing or Nonreducing End of the Tumor Antigen

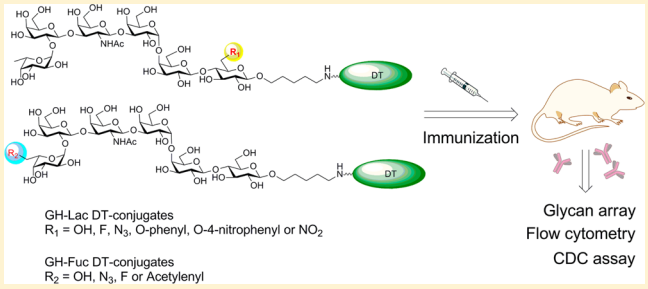
Hsin-Yu Lee,[†,‡] Chien-Yu Chen,[†] Tsung-I Tsai,[†] Shiou-Ting Li,[†] Kun-Hsien Lin,[†] Yang-Yu Cheng,[†] Chien-Tai Ren,[†] Ting-Jen R. Cheng,[†] Chung-Yi Wu,*[,†] and Chi-Huey Wong*[,†,‡]

[†]Genomics Research Center, Academia Sinica, 128 Academia Road, Section 2, Nankang, Taipei 115, Taiwan

[‡]Department of Chemistry, National Taiwan University, 1 Roosevelt Road, Section 4, Taipei, 106, Taiwan

Supporting Information

ABSTRACT: Globo H-based therapeutic cancer vaccines have been tested in clinical trials for the treatment of late stage breast, ovarian, and prostate cancers. In this study, we explored Globo H analogue antigens with an attempt to enhance the antigenic properties in vaccine design. The Globo H analogues with modification at the reducing or nonreducing end were synthesized using chemoenzymatic methods, and these modified Globo H antigens were then conjugated with the carrier protein diphtheria toxoid cross-reactive material (CRM) 197 (DT), and combined with a glycolipid C34 as an adjuvant designed to induce a class switch to form the vaccine candidates. After Balb/c mice injection, the immune response was studied by a glycan array and the results showed that modification at the C-6 position of reducing end glucose of Globo H with the fluoro, azido, or phenyl group elicited IgG antibody response to specifically recognize Globo H (GH) and the GH-related epitopes, stage-specific embryonic antigen 3 (SSEA3) (also called Gb5) and stage-specific embryonic antigen 4 (SSEA4). However, only the modification of Globo H with the azido group at the C-6 position of the nonreducing end fucose could elicit a strong IgG immune response. Moreover, the antibodies induced by these vaccines were shown to recognize GH expressing tumor cells (MCF-7) and mediate the complement-dependent cell cytotoxicity against tumor cells. Our data suggest a new potential approach to cancer vaccine development.



INTRODUCTION

Tumor associated carbohydrate antigens (TACAs) are overexpressed on the surface of cancer cells and related to tumor cell adhesion and metastasis.[1] Thus, TACAs are potential targets for cancer vaccine development.[2] However, most TACAs have poor immunogenicity and many approaches have been developed to increase the immune response of carbohydrate-based vaccines, including conjugation with a carrier protein,[3] administration with an immunologic adjuvant,[4] using unnatural glycosidic linkage,[5] clustered antigens,[6] unimolecular polyvalent vaccine,[7] or heteroglycan multivalent vaccine.[8] Using these strategies, a few carbohydrate-based vaccines that could elicit significant immune responses to target glycan structures were designed for cancer therapy and entered clinical trials.[3,9] Among them, the clinical trials of Theratope and GMK with adjuvant QS-21 failed to produce statistically significant difference between time-to-disease and overall survival rate. Probably these two vaccines could not elicit robust T cell-dependent immune response in patients.[10] Specifically, Theratope and GMK induced a higher level of IgM in patients but could not induce a strong immune IgG response, which is a major problem in carbohydrate-based vaccine development.[11]

Globo H (GH; Fucα1 → 2Galβ1 → 3GalNAcβ1 → 3Galα1 → 4Galβ1 → 4Glc) is a member of the globo series glycosphingolipids (Figure 1). It was first found and characterized in human teratocarcinoma cells and breast cancer MCF-7 cells in 1983,[12] and was subsequently found overexpressed in many types of human cancer cells including breast, prostate, ovary, pancreas, brain, endometrium, gastric, colon, and lung cancers.[13] A Globo H vaccine using KLH as carrier and QS-21 as adjuvant prepared by Livingston and Danishefsky showed a positive result in a phase I study against metastatic breast cancer patients.[14] With improvement in synthesis,[15] it is now in a phase III clinical trial in Taiwan and a phase II clinical trial in the United States, Korea, Hong Kong, and India for late stage breast cancer patients and in a phase II clinical trial for ovarian cancer patients in Taiwan. However, these early stage clinical results showed that the induced IgM antibodies were still much higher than IgG antibodies.[14,16] Recently, our group has developed a better vaccine using diphtheria toxoid cross-reactive material (CRM) 197 (DT) as carrier and a glycolipid C34 as adjuvant to induce a class switch with robust IgG antibody response against GH and the GH-related epitopes, stage-specific embryonic antigen 3 (SSEA3) (also called Gb5)

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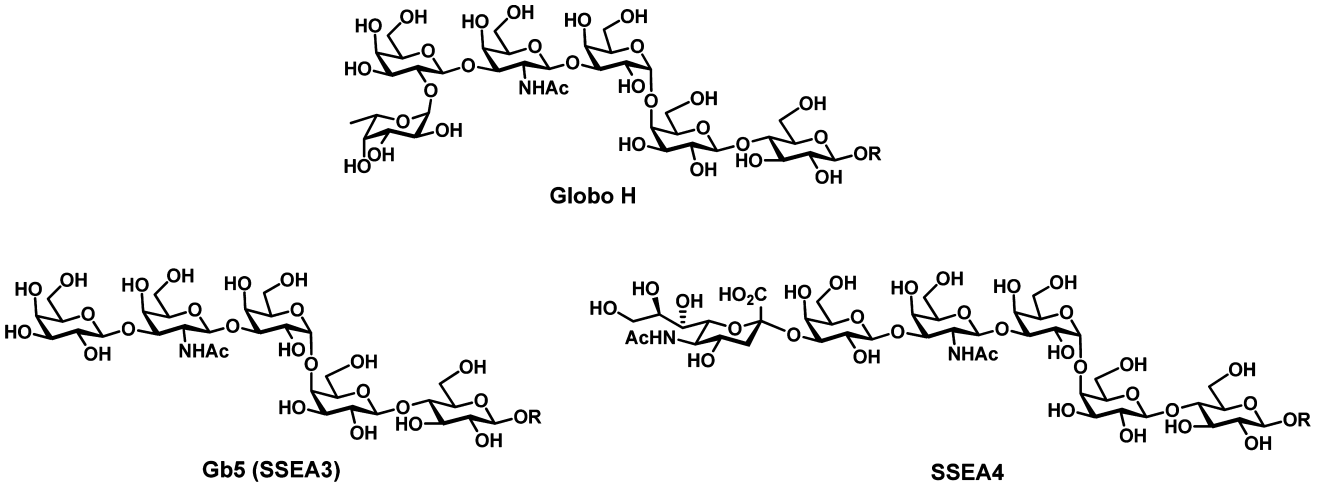
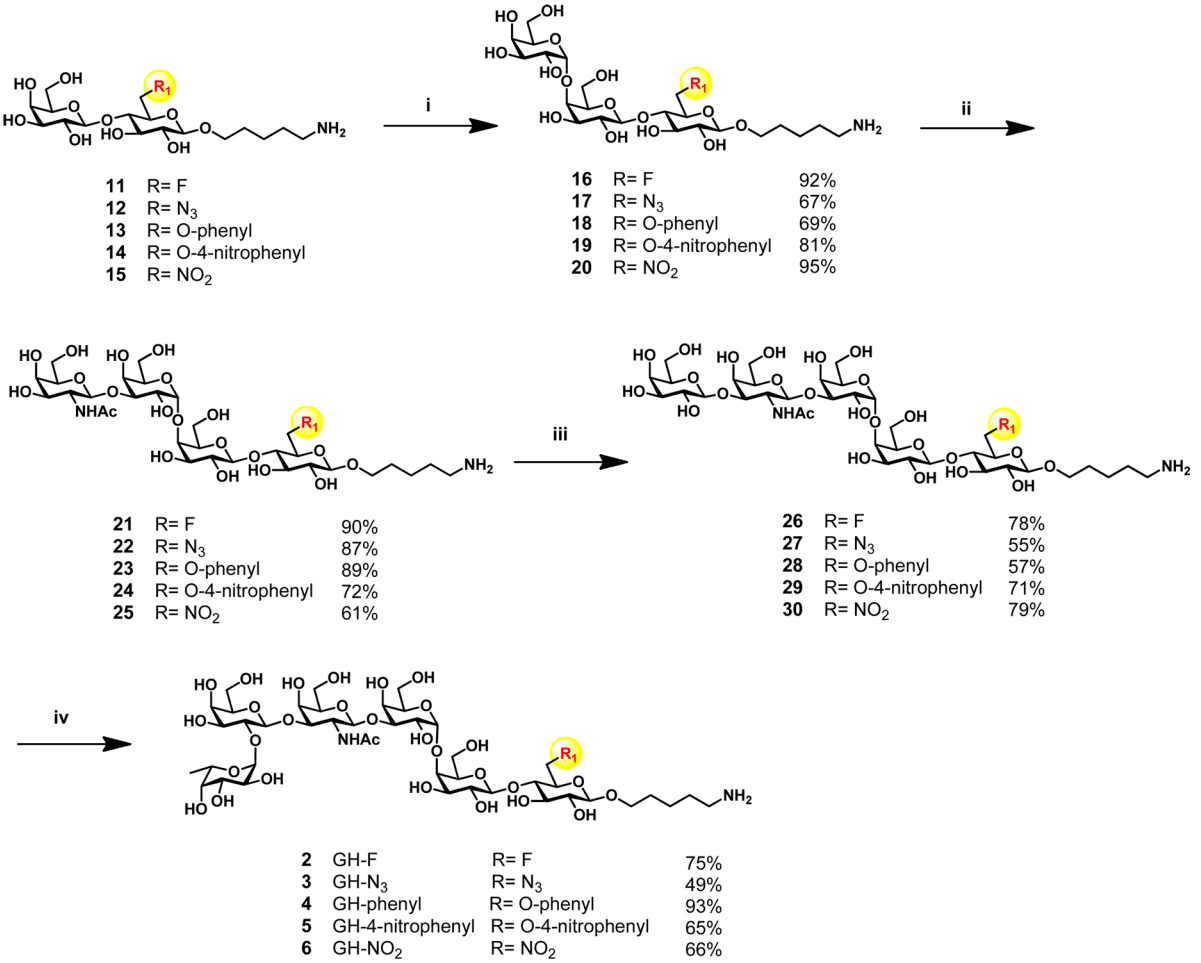


Figure 1. Structures of the globo series glycosphingolipisd Globo H, Gb5 (SSEA3), and SSEA4 (R = ceramide).

Scheme 1. Chemoenzymatic Synthesis of GH-Lac Analogues[a]



[a](i) Galactose, ATP, UTP, Mg^{2+}, Tris buffer (pH 7.0), GalK, AtUSP, LgtC, PK, and PPA; (ii) *N*-acetylgalactose, ATP, UTP, Mg^{2+}, Tris buffer (pH 7.0), GlmU, NahK, LgtD, PK, and PPA; (iii) galactose, ATP, UTP, Mg^{2+}, Tris buffer (pH 7.0), GalK, AtUSP, LgtD, PK, and PPA; (iv) fucose, ATP, GTP, Mg^{2+}, Tris buffer (pH 7.0), FKP, FutC, PK, and PPA. Enzymes: GalK, galactokinase; AtUSP, UDP-sugar pyrophosphorylase; LgtC, α1,4-galactosyl- transferase; PK, pyruvate kinase; PPA, inorganic pyrophosphatase; GlmU, *N*-acetyl glucosamine-1-phosphate uridyltransferase; NahK, *N*-acetyl- hexosamine kinase; LgtD, β1,3-*N*-acetylgalactosaminyltransferase; FKP, bifunctional fucokinase/GDP-L-fucose pyrophosphorylase; FutC, α-1,2-fucosyltransferase. Compounds: ATP, adenosine-5′- triphosphate; UTP, uridine-5′-triphosphate; GTP, guanosine-5′-triphosphate.

and stage-specific embryonic antigen 4 (SSEA4), all found on breast cancer cells and the cancer stem cells only (Figure 1).[13b]

Previous studies showed that modification of carbohydrate antigen structures (MCAS) could effectively elicit a higher level

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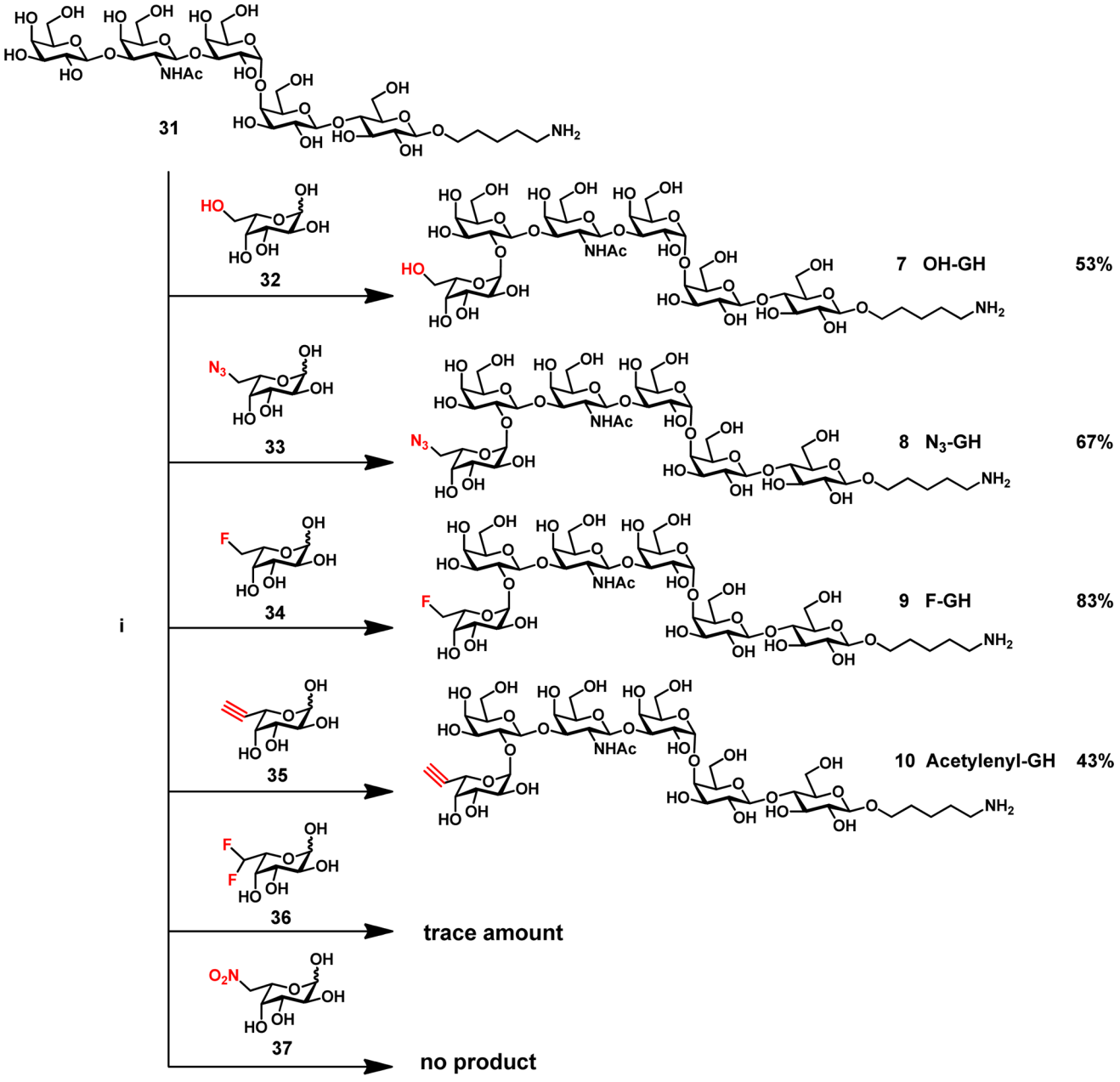
Scheme 2. Chemoenzymatic Synthesis of GH-Fuc Analogues[a]



[a]Reagents and conditions: (i) FKP, Fut C, PPA, PK, Mg^{2+}, ATP, GTP, and Tris buffer (pH 7.0).

of immune response.[17] For example, in the modification study of the capsular polysaccharide of group B meningococci, the *N*-acetyl groups of α-(2,8)-linked polysialic acid (PSA) was replaced with the *N*-propinoyl group and such a modification elicited a high antibody response to recognize not only the *N*-propinoyl PSA, but also the native *N*-acetyl PSA.[18] Similar approaches were applied to STn[19] and GM3[20] antigens to produce high antibody titers against modified and native antigens. The results indicated that *N*-phenylacetyl, N_3, *N*-fluoroacetyl, or *N*-difluoroacetyl modifications on glycan antigens could improve the immunogenicity.[19a,c] These nonself antigens were found to be more immunogenic. In addition, the sizes and hydrophobicity of *N*-phenylacetyl structures may make them more immunogenic.[19a,b,20] The N_3 property on the glycan may play a critical role to induce more antibody response.[19c] The immunogenicity of fluorinated modifications might be advantageous due to the similar atom radius and the lipophilicity of the fluorine atom compared to the hydrogen atom.[19c,21] Moreover, the Schultz group reported that incorporation of a *p*-nitrophenylalanine into the tumor necrosis factor-α (TNF-α) could break immune tolerance and induce more antibody response to TNF-α.[22] Using functional group modified glycans as antigens, although some progress has been achieved, most cases are the *N*-modification of disaccharide (STn), trisaccharide (GM3), and polysialic acid (PSA) and some are based on fluorinated MUC1 glycopeptide antigens.[18a,19a,d,20,21,23] There is lack of a general strategy for the preparation of carbohydrate-based vaccines to induce IgG response with a long-term memory. The systematic modification on reducing and nonreducing end of oligosaccharides was never been done before. In this study, we systematically modified the C-6 position of Glc in the lactose (Lac) moiety at

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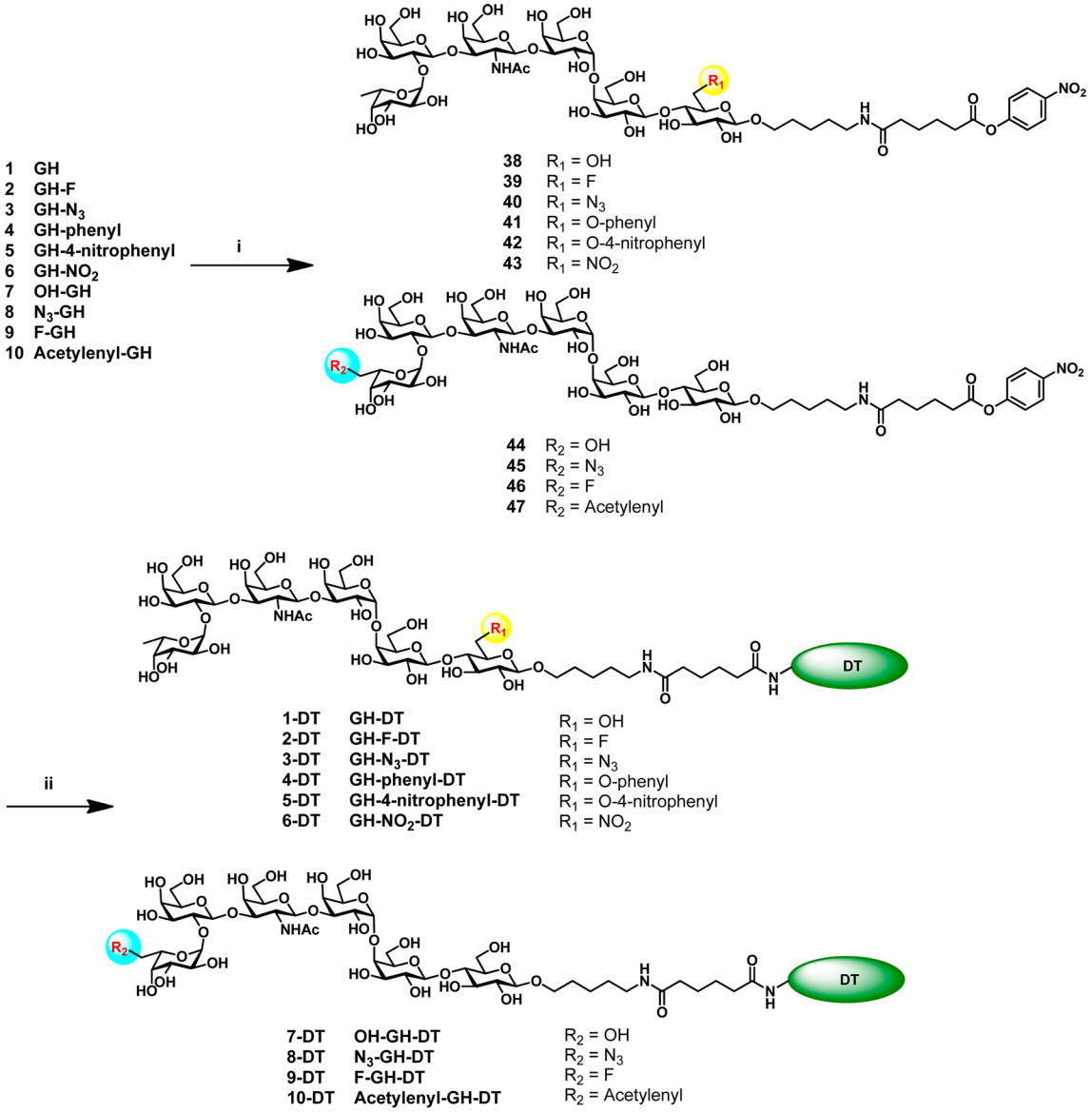
Scheme 3. Synthesis of GH-Lac and GH-Fuc Modified Vaccines[a]



[a]Reagents and conditions: (i) *p*-nitrophenyl adipate diester, DMF, and Et_3N; (ii) DT and PBS buffer (pH 7.8).

the reducing end or the C-6 position of fucose (Fuc) at the nonreducing end of GH. The resulting unnatural sugar moieties were then conjugated with DT to form GH Lac and Fuc analogues-DTs for immunogenicity studies in the Balb/c mouse model. The results were analyzed by a glycan array and showed that modification of the nonreducing end of Globo H with the azido group and the reducing end of Globo H with the fluoro, azido, or phenyl group elicited strong IgG antibodies in the presence of C34 to specifically recognize Globo H and the GH-related epitopes, Gb5 and SSEA4. The antibodies induced by theses vaccines were shown to recognize GH expressing tumor cells (MCF-7) and mediate the complement-dependent cell cytotoxicity against tumor cells.

RESULTS AND DISCUSSION

GH is commonly synthesized using the glycal strategy,[24] trichloroacetimidate method,[25] two directional glycosylation strategy,[26] linear synthesis,[27] automated solid-phase synthesis,[28] multicomponent one-pot synthesis,[29] and programmable one-pot synthesis.[15b,30] However, chemical synthesis methods often require multiple protection and deprotection steps, resulting in relatively low yields. An alternative efficient strategy was based on the use of enzymes[31] coupled with effective sugar nucleotide regeneration.[15a] Using this method,[15a] the GH-Lac and GH-Fuc analogues were easily prepared for this study using glycosyltransferases (LgtC, LgtD, FutC) and cofactor regeneration systems (UDP-Gal, UDP-GalNAc, GDP-Fuc). The starting Lac analogues **11**−**15** and the Fuc analogues **32**−**37** were synthesized by chemical methods (Schemes S1−S5 in the Supporting Information (SI)).

The synthesis of the GH-Lac analogues **2**−**6** (reducing end analogues) (Scheme 1) was started from the Lac analogues **11**−**15** following the enzymatic procedure described previously.[15a] The Gb3-Lac analogues **16**−**20** were synthesized with galactose, α1,4-galactosyltransferase (LgtC), and the UDP-Gal regeneration system including UDP-sugar pyrophosphorylase (AtUSP), galactokinase (GalK), pyruvate kinase (PK), and inorganic pyrophosphatase (PPA). LgtC has been carefully

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Table 1. MALDI-TOF Analysis of Average Carbohydrate Incorporation

glycoconjugate	after glycosylation[a]	(n) average incorporation	carbohydrate percentage
(**1-DT**) GH-DT	66 943	7.10	12.9%
(**2-DT**) GH-F-DT	67 406	7.47	13.4%
(**3-DT**) GH-N_3-DT	66 505	6.60	12.2%
(**4-DT**) GH-phenyl-DT	66 057	5.99	11.7%
(**5-DT**) GH-4-nitrophenyl-DT	67 588	6.94	13.7%
(**6-DT**) GH-NO_2-DT	66 119	6.12	11.7%
(**7-DT**) OH-GH-DT	64 308	4.86	9.3%
(**8-DT**) N_3-GH-DT	64 742	5.11	9.9%
(**9-DT**) F-GH-DT	68 869	8.56	15.3%
(**10-DT**) acetylenyl-GH-DT	65 881	6.17	11.5%

[a]Peak m/z in MALDI-TOF.

characterized and utilized in the synthesis of α-(1→4)-galactosylated analogues.[32] Here, LgtC was also found to exhibit good activities to the Lac analogues (**11**−**15**). The yields of Gb3-F **16**, Gb3-phenylNO_2 **19**, and Gb3-NO_2 **20** were 92, 81, and 95%, respectively, and the yields of Gb3-N_3 **17** and Gb3-phenyl **18** were 67 and 69%, respectively.

The Gb3-Lac analogues **16**−**20** were used as acceptors for the synthesis of the Gb4 analogues **21**−**25** using *N*-acetylgalactosamine, β1,3-*N*-acetylgalactosaminyltransferase (LgtD), and the UDP-GalNAc regeneration system including *N*-acetylhexosamine kinase (NahK), *N*-acetyl glucosamine-1-phosphate uridyltransferase (GlmU), pyruvate kinase (PK), and inorganic pyrophosphatase (PPA).[15a] After overexpression and biochemical characterization,[33] LgtD was used to glycosylate Gb3-F **16**, Gb3-N_3 **17**, and Gb3-phenyl **18** as acceptors to obtain Gb4-F **21**, Gb4-N_3 **22**, and Gb4-phenyl **23** in 90, 87, and 89% yields, respectively. From Gb3-phenylNO_2 **19** and Gb3-NO_2 **20**, Gb4-phenylNO_2 **24** and Gb4-NO_2 **25** were obtained in 72 and 61% yields, respectively.

The Gb5-Lac analogues **26**−**30** were obtained from the Gb4 analogues **21**−**25** and galactose using β1,3-*N*-acetylgalactosaminyltransferase (LgtD) and the UDP-gal regeneration system as described before.[15a] Gb5-F **26**, Gb5-N_3 **27**, Gb5-phenyl **28**, Gb5-phenylNO_2 **29**, and Gb5-NO_2 **30** were obtained in 55−79% yields.

The GH-Lac analogues **2**−**6** were synthesized from the Gb5-Lac analogues **26**−**30** using α-1,2-fucosyltransferase (FutC), bifunctional fucokinase/GDP-L-fucose pyrophosphorylase (FKP), pyrophosphatase (PPA), pyruvate kinase (PK,) and fucose.[15a] GH-F **2** and GH-phenyl **4** were prepared from acceptors Gb5-F **26** and Gb5-phenyl **28** in 75 and 93% yields, respectively. Using Gb5-N_3 **27**, Gb5-phenylNO_2 **29**, and Gb5-NO_2 **30** as acceptors GH-N_3 **3**, GH-phenylNO_2 **5**, and GH-NO_2 **6** were obtained in 49, 65, and 66% yields, respectively.

The synthesis of GH-Fuc analogues **7**−**10** (nonreducing end analogues) (Scheme 2) also followed the method previously described[15a] by combining the fucose analogue and the acceptor Gb5 oligosaccharide with recombinant FKP, α-1,2-fucosyltransferase (FutC), PPA, and PK. The starting material Gb5 oligosaccharide with pentyl amine **31** was synthesized using a chemical method described previously.[30] Using this chemoezymatic method, a series of GH-Fuc analogues **7**−**10** was synthesized in 43−83% yields. Although compound **36** was reacted with FKP to form GDP-**36**, it was not a suitable donor for FutC and a trace amount of the product was formed. In addition, compound **37** is not a substrate for FKP, and GDP-**37** intermediate was not formed.

The structures of all purified GH analogues and truncated forms were confirmed by nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry (HRMS) for further use.

To synthesize GH-Lac and GH-Fuc DT-conjugates (**1-DT** to **10-DT**), the amine-terminated GH Lac analogues **1**−**6** or GH Fuc analogues **7**−**10** were reacted with the homobifunctional *p*-nitrophenyl linker to afford the corresponding half esters **38**−**47** in 61−86% yields (Supporting Information).[5b,34] After purification by reverse phase chromatography, the half esters and DT were coupled in PBS buffer (pH 7.8) overnight (Scheme 3). The number of GH analogues incorporated into DT was characterized by MALDI-TOF MS (Table 1).

To investigate the immunogenicity of the GH analogues DT-conjugates (**1-DT** to **10-DT**), 10 groups of 5 female BALB/c mice were immunized intramuscularly with GH analogues-DT (2 μg GH analogues) and 2 μg of the glycolipid adjuvant C34 three times at biweekly intervals. Our previous result showed that the glycoconjugate vaccine with the α-galactosylceramide analogue C34 as an adjuvant exhibited a better immunization result to induce higher IgG antibodies titer than the glycoconjugate vaccine with other adjuvants such as QS-21, aluminum phosphate, or MF59.[13b] Moreover, the anti-GH antibodies titer was low with GH-protein conjugates alone without any adjuvants.[13b] The antisera from each immunogen were obtained 10 days after the third immunization and were tested on the glycan microarray containing 94 chemically synthesized glycans, including GH **1**, GH analogues **2**−**10**, GH analogues fragments **11**−**30** and other tumor-associated carbohydrate antigens (Table S2 in the SI). Because some chemical modifications were carried out on the glycan, some functional linkers were also included in the glycan array to check the cross-reactivity. The glycan arrays were incubated with the sera diluents (1:200 dilution to 1:20 000 dilution) at 4 °C for 1 h, and then excess sera antibodies were washed out. The glycan arrays were incubated with secondary antibodies (fluorescently labeled goat anti-mouse IgG or IgM antibodies) at 4 °C in the dark for 1 h. Finally, the arrays were then washed three times and scanned at 635 nm wavelength with a microarray fluorescence chip reader. The results were based on the RFU values correlated with the relative amounts of antibodies to recognize the glycans

Antibodies induced by the GH analogues DT-conjugates (**1-DT** to **10-DT**) were specifically recognized by GH, GH analogues and GH fragments but not by other TACAs and functional linkers (Figures S6−S15 in the SI). In the previous study, we found that GH, Gb5 and SSEA4 are overexpressed not only on breast cancer cells but also on breast cancer stem

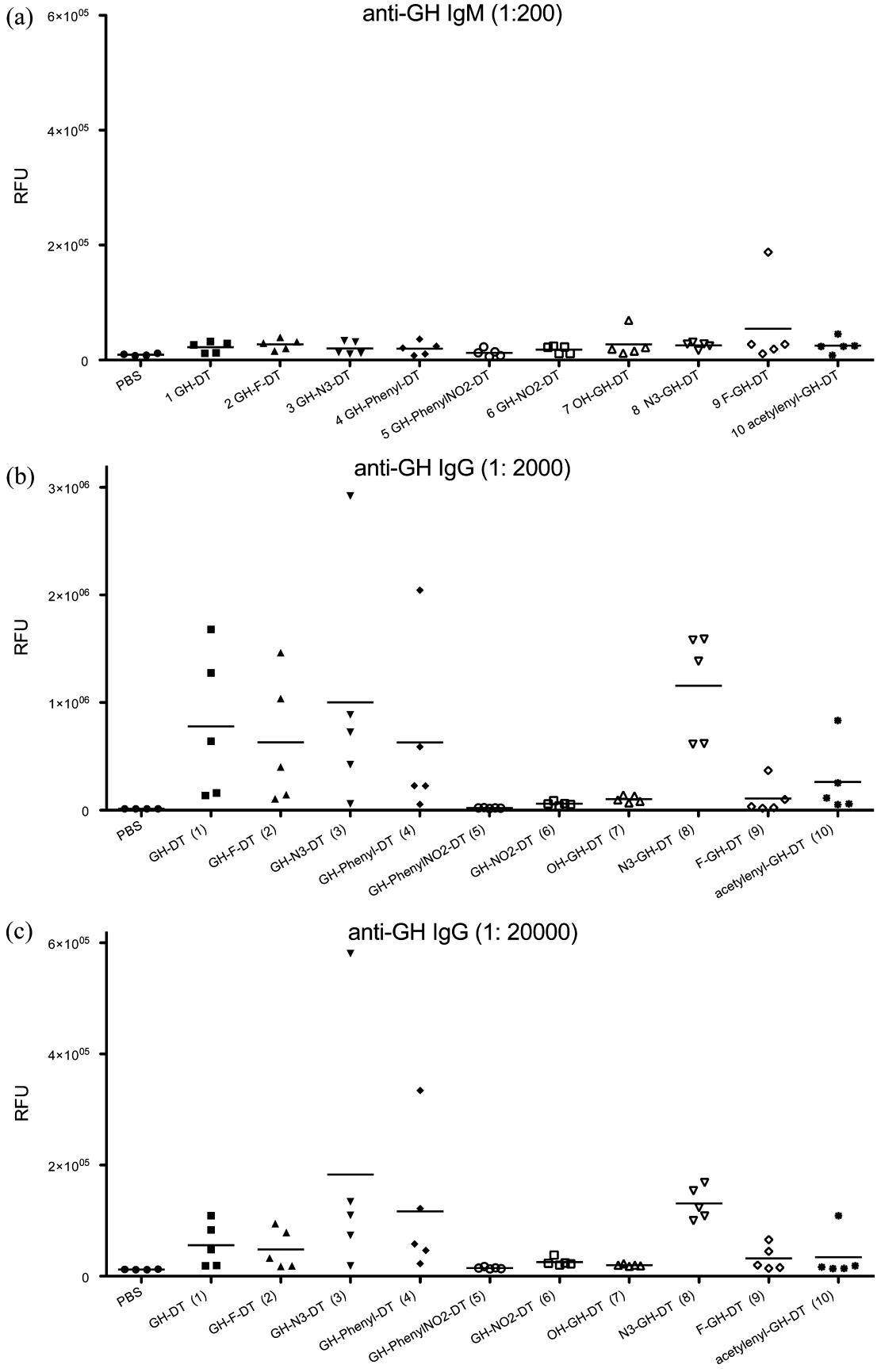

Figure 2. continued

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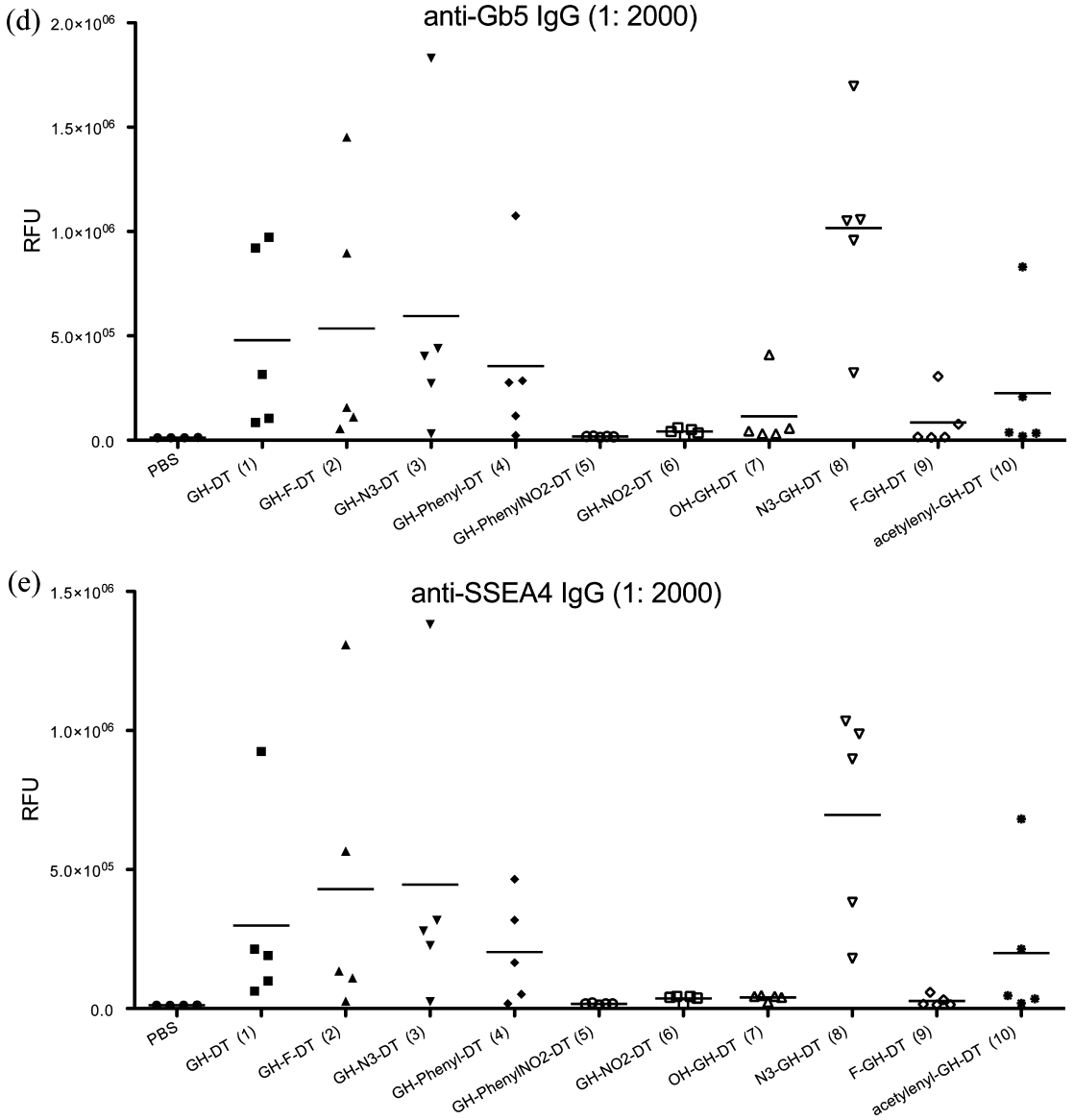

Figure 2. Antibodies elicited by GH-analogue DT conjugates against (a) IgM level against GH (1:200 dilution) (b) IgG level against GH (1:2000 dilution), (c) IgG level against GH (1:20 000 dilution) (a statistically significant difference $^*P < 0.05$ was obtained by Student's *t* test between GH-DT (**1-DT**) versus N_3-GH-DT (**8-DT**)), (d) IgG level against Gb5 (1:2000 dilution), and (e) IgG level against SSEA4 (1:2000 dilution). Groups of five Balb/c mice were immunized intramuscularly with GH analogues-DT (2 μg of GH analogues) and 2 μg of glycolipid C34 as the adjuvant. All antisera were collected 10 days after the third immunization and analyzed by a glycan array. Each data point represents the total fluorescence intensity for an individual mouse after third immunizations, and the horizontal lines indicate the mean for the group of five mice.

cells and the core structures of them are similar (Galβ1 → 3GalNAcβ1 → 3Galα1 → 4Galβ1 → 4Glc).[13b,35a] Therefore, GH and the GH-related epitopes, Gb5 and SSEA4 were selected as standard antigens for all DT-conjugates (Figure 2). The sera obtained from these glycoconjugates induced high IgG antibodies titer, indicating a T-cell-dependent immune response. Interestingly, no significant IgM production was observed for all GH-Lac or Fuc analogues (Figure 2a, and Figure S4 in the SI). Regarding the IgG level against GH (1:2000 dilution), the titers of antibodies induced by GH-N_3-DT (**3-DT**), N_3-GH-DT (**8-DT**), GH-F-DT (**2-DT**), and GH-phenyl-DT (**4-DT**) were comparable to the native antigen GH-DT conjugate (**1-DT**) (Figure 2b). In 1:20 000 dilution, the titer of antibodies induced by N_3-GH-DT (**8-DT**) was, however, significantly higher than that of the native antigen GH-DT conjugate (**1-DT**) (Figure 2c). The azido group appears to be an immune modulator as GH-N_3-DT (**3-DT**) and N_3-GH-DT (**8-DT**) provide good titers. The reason for the enhancement of immunogenicity is unknown, but the N_3 property on the glycan of GH-N_3 **3** or N_3-GH **8** compared to native GH may play a critical role. The immunogenicity modulation by the fluoro (F) group on GH is regioselective.[19c,36] The F moiety at the C-6 position of Glc at the reducing end of GH could induce comparable titer to native GH, but the titer induced by the F group at the C-6 position of Fuc at the nonreducing end of GH showed a lower reaction with GH. Interestingly, antibodies induced by GH-phenyl-DT (**4-DT**) can cross-react with GH. This cross-immunogenicity is inconsistent with the previous report that no cross-reaction with native GM3 or STn was found with the use of *N*-phenylacetyl GM3 or STn based vaccines.[19a,20] The immunogens GH-phenylNO_2-DT (**5-DT**), GH-NO_2-DT (**6-DT**),

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OH-GH-DT (**7-DT**), F-GH-DT (**9-DT**), and acetylenyl-GH-DT (**10-DT**) gave weak response to GH. Moreover, GH-phenylNO$_2$-DT (**5-DT**) and GH-NO$_2$-DT (**6-DT**) elicited strong immune response to the phenylNO$_2$ and the NO$_2$ sugar analogues but not to the native antigen GH analogues (Figures S10 and S11 in the SI). This result showed that phenylNO$_2$ and NO$_2$ are strong immunogenic groups that suppress the immune response to induce antibodies to recognize the native antigens Globo H and the GH-related epitopes, Gb5 and SSEA4. Interestingly, antibodies induced by these glycoconjugates also showed the same pattern in recognizing Gb5 and SSEA4 (Figure 2d and e). Therefore, we concluded that modification at the C-6 position of reducing end glucose of Globo H with the fluoro, azido, or phenyl group elicited IgG antibody response to specifically recognize Globo H, Gb5, and SSEA4. However, only the modification of Globo H with the azido group at the C-6 position of the nonreducing end fucose could elicit strong IgG immune response.

The ratio of IgG versus IgM glycan specific antibodies is another important factor for the evaluation of vaccine efficacy. After the third immunization, the ratio of anti-GH IgG/IgM (1:200 dilution) of GH-DT (**1-DT**) is 75.80. For GH-F-DT (**2-DT**), GH-N$_3$-DT (**3-DT**), GH-phenyl-DT (**4-DT**), and N$_3$-GH-DT (**8-DT**), the ratios of anti-GH IgG/IgM are 78.07, 68.86, 83.57, and 95.29, respectively. Moreover, the immunogens GH-phenylNO$_2$-DT (**5-DT**), GH-NO$_2$-DT (**6-DT**), OH-GH-DT (**7-DT**), F-GH-DT (**9-DT**), and acetylenyl-GH-DT (**10-DT**) gave low ratios of anti-GH IgG/IgM.

Table 2. Ratio of Anti-GH IgG versus IgM after the Third Immunization with Different Glycoconjugates

glycoconjugate	IgG(1:200)[a]	IgM(1:200)[a]	IgG/IgM
(**1-DT**) GH-DT	1 921 346	25 349	75.80
(**2-DT**) GH-F-DT	2 134 265	27 337	78.07
(**3-DT**) GH-N$_3$-DT	1 386 958	20 141	68.86
(**4-DT**) GH-phenyl-DT	1 917 407	22 945	83.57
(**5-DT**) GH-4-nitrophenyl-DT	34 575	12 448	2.78
(**6-DT**) GH-NO$_2$-DT	149 817	18 276	8.20
(**7-DT**) OH-GH-DT	640 634	27 540	23.26
(**8-DT**) N$_3$-GH-DT	2 343 602	24 594	95.29
(**9-DT**) F-GH-DT	615 074	54 523	11.28
(**10-DT**) acetylenyl-GH-DT	720 516	23 634	30.49

[a]Anti-GH antibody titers are presented as the median for groups of five mice. The glycan arrays were incubated with the sera diluents (1:200 dilution) at 4 °C for 1 h, and then excess sera antibodies were washed out. The glycan arrays were incubated with secondary antibodies (fluorescently labeled goat anti-mouse IgG or IgM antibodies) at 4 °C in the dark for 1 h. Finally, the arrays were then washed three times and scanned at 635 nm wavelength with a microarray fluorescence chip reader. The result was based on the RFU values correlated with the relative amounts of antibodies that recognized the glycans.

After the analysis of glycan array, we found that antibodies induced by GH-DT (**1-DT**) recognized the GH-N$_3$ (**3**), N$_3$-GH (**8**), GH-phenyl (**4**), and GH-phenylNO$_2$ (**5**) antigens much higher than the native GH antigen (**1**) (Figure S6, SI). Vice versa, the results also showed that antibodies induced by GH-N$_3$-DT (**3-DT**) recognized the GH-N$_3$ (**3**), N$_3$-GH (**8**), GH-phenyl (**4**), and GH-phenylNO$_2$ (**5**) antigens much higher than the native GH antigen (**1**) (Figure S8, SI). Similar results are also found for the N$_3$-GH-DT (**8-DT**) (Figure S13, SI). We observed that most of anti-carbohydrate antibodies induced by glycoconjugates recognized more the glycan-mimic structure antigens than the native glycan antigen. The possible reason for the observation may come from that the modified functional groups of native glycan antigen provided another binding ability to increase the antibody binding. Interestingly, antibodies induced by GH-phenyl-DT (**4-DT**) mainly recognized the GH-phenyl (**4**) and GH-phenylNO$_2$ (**5**), followed by GH-N$_3$ (**3**) and N$_3$-GH (**8**) and then native GH antigen (**1**) (Figure S9, SI).

Further glycan array analysis of the antibody isotypes of the IgG subclasses of antisera from these vaccines showed that the antibodies have a significant amount of IgG1, IgG2b, IgG2c, and IgG3 and low level of IgG2a (Figure S5, SI). Specifically, the presence of IgG3 antibodies in the antisera is a typical anticarbohydrate response and consistent with a T cell-mediated immunity.[37]

The capabilities of the mouse antisera induced by GH-DT (**1-DT**), GH-F-DT (**2-DT**), GH-N$_3$-DT (**3-DT**), GH-phenyl-DT (**4-DT**), and N$_3$-GH-DT (**8-DT**) to recognize the GH-positive MCF7 human breast cancer cells were examined by flow cytometry (Figure 3). As expected, the antiserum elicited by GH-DT (**1-DT**) was significantly reactive with GH-positive MCF7 cells compared with the antisera from untreated mouse. MCF7 cells were also specifically recognized by the antisera elicited by GH analogues-DT (**2-DT**, **3-DT**, **4-DT**, and **8-DT**). In contrast, GH-negative A375 melanoma cells were not recognized by the antisera elicited by GH-DT (**1-DT**) and GH analogues-DT (**2-DT**, **3-DT**, **4-DT**, and **8-DT**) (Figure S18, SI).

Complement-dependent cytoxicity (CDC) was studied by GH-expressing MCF7 cancer cells (Figure 4). In comparison with rabbit complement plus the sera from untreated mouse, the antisera obtained from immunization with GH-DT (**1-DT**), GH-F-DT (**2-DT**), GH-N$_3$-DT (**3-DT**), GH-phenyl-DT (**4-DT**), and N$_3$-GH-DT (**8-DT**) were able to significantly induce cancer cell cytotoxicity. The cell cytotoxicity of the antisera obtained from GH-phenyl-DT (**4-DT**) or N$_3$-GH-DT (**8-DT**) was comparable to the native antigen GH-DT (**1-DT**). Interestingly, the antisera derived from GH-F-DT (**2-DT**) or GH-N$_3$-DT (**3-DT**) vaccine could induce near 5% higher cancer cell cytotoxicity comparing to GH-DT (**1-DT**).

In conclusion, this study has established a strategy for the chemoenzymatic synthesis of GH analogues and their protein conjugates. The immunological properties of GH analogue conjugates were evaluated using a glycan array and compared to the native antigen GH-DT (**1-DT**). The results showed that modification at the C-6 position of the reducing end of Globo H with the fluoro, azido, or phenyl group elicited IgG antibody response to specifically recognize Globo H and the GH-related epitopes, Gb5 and SSEA4. However, only the modification of Globo H with the azido group at the C-6 position of the nonreducing end fucose could elicit a strong IgG immune response. Moreover, antibodies induced by GH-DT (**1-DT**), GH-F-DT (**2-DT**), GH-N$_3$-DT (**3-DT**), GH-phenyl-DT (**4-DT**), and N$_3$-GH-DT (**8-DT**) recognized GH expressing tumor cells (MCF-7) and could mediate the complement-dependent cell cytotoxicity against tumor cells. Our data suggest a new potential approach for a new generation of vaccines based on modification of carbohydrate antigen structures.

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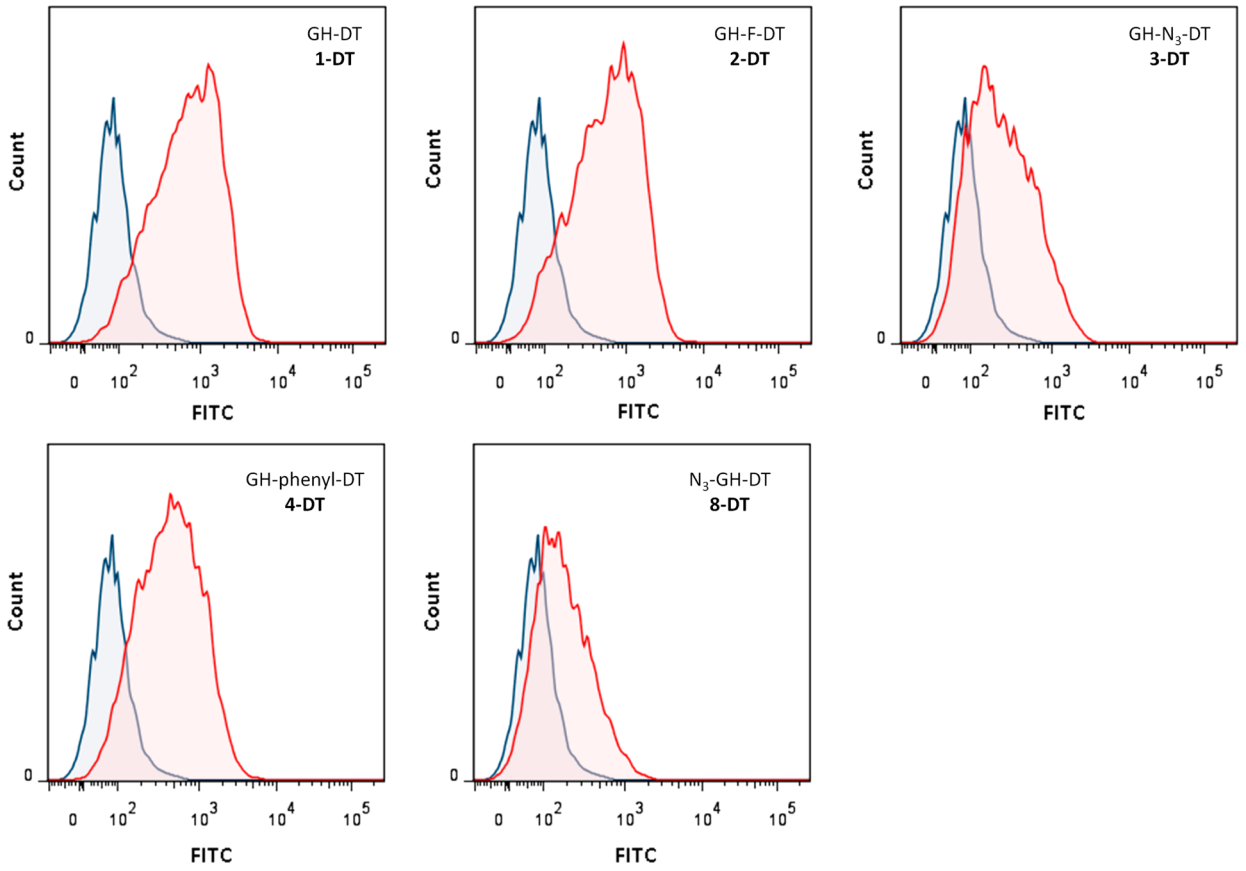

Figure 3. GH-analogue DT-conjugate-induced mouse antibodies recognize GH-positive breast cancer cell line (MCF-7). Flow cytometry histograms for cells stained with serum from untreated mouse (blue) and the third immunization antiserum (red) collected from GH-DT (**1-DT**), GH-F-DT (**2-DT**), GH-N_3-DT (**3-DT**), GH-phenyl-DT (**4-DT**), or N_3-GH-DT (**8-DT**). The binding was detected by flow cytometry using FITC-labeled anti-mouse IgG/IgM antibody.

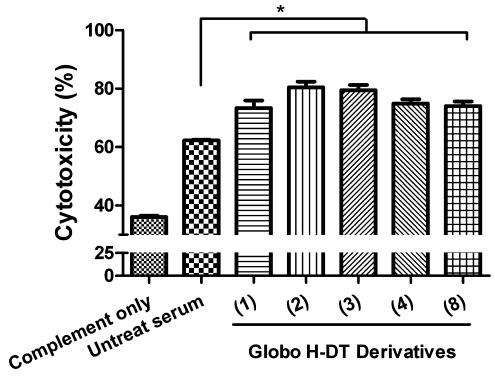

Figure 4. Antibodies elicited by GH analogues mediate complement-dependent cytotoxicity (CDC) to eliminate GH-expressing tumor cells. MCF-7 cells were incubated with rabbit complement only or rabbit complement with serum from untreated mouse, or antiserum from mice vaccinated with GH-DT **1-DT** (1), GH-F-DT **2-DT** (2), GH-N_3-DT **3-DT** (3), GH-Phenyl-DT **4-DT** (4), and N_3-GH-DT **8-DT** (8) at 37 °C for 2 h, the cytotoxicity induced by the serum or antiserum was then determined using the LDH assay. *$P < 0.05$ was obtained by Student's t test.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

General methods, supplementary schemes, synthetic protocols, spectroscopic and analytical data for new compounds, and additional biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

cyiwu@gate.sinica.edu.tw
chwong@gate.sinica.edu.tw

Notes

The authors declare no competing financial interest.

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