J|A|C|S COMMUNICATIONS

Published on Web 06/24/2009

Immunological Response from an Entirely Carbohydrate Antigen: Design of Synthetic Vaccines Based on Tn−PS A1 Conjugates

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The introduction of vaccines into medical practice has been one of the most significant advancements of modern medicine.[1] Its success can be attributed to the fact that vaccinology has expanded beyond its traditional mainstays (attenuated or dead microorganisms, inactivated bacterial toxins, and protein subunit vaccines) to the likes of recombinant proteins and glycoproteins, synthetic peptides, and conjugate vaccines.[2] Despite the enormous success, there remains a need for effective vaccines for the treatment of serious diseases such as malaria, AIDS, antibiotic-resistant infections, and cancer.

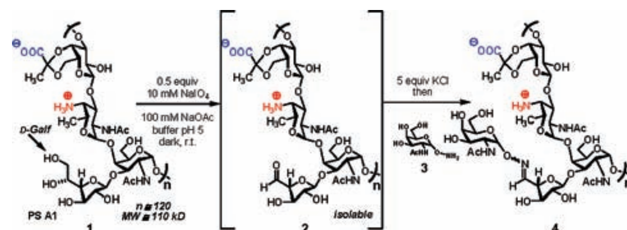
Carbohydrates are involved in a wide variety of biological roles.[3] For example, oncogenic transformation of cells is closely correlated with dramatic changes in their glycosylation patterns.[4] Aberrant polysaccharides expressed on these cancer-cell surfaces [so-called tumor-associated carbohydrate antigens (TACAs)] have been used successfully in the diagnosis and prognosis of cancer for many years.[4b] Furthermore, TACAs can induce changes in antigenicity and immunogenicity in cancer cells, which may allow TACA-derived cancer vaccines and cancer immunotherapies to be realized.[4c]

Although isolated/pure carbohydrates/polysaccharides have long been known to be T-cell-independent and exhibit poor immunogenicity,[5] glycoconjugate vaccines, in which carbohydrate antigens are tethered to strong immunogenic carrier proteins, such as bovine serum albumin (BSA), tetanus toxoid, keyhole limpet hemocyanin, and others, can elicit an MHCII T-cell response characterized by immunoglobulin G (IgG) production.[6] Extensive research has focused on the creation of synthetic vaccines[7] based on complex carbohydrate antigens/epitopes conjugated to these carriers. Such vaccines have shown varying degrees of promise, and as an outcome, the components necessary for eliciting protective antibody production have become more defined.[8] Paradoxically, these conjugate vaccines can be less intrinsically immunogenic, even as they become safer and more precisely targeted. This dichotomy typically arises from nonrelated binding and a strong immune response against the carrier protein, resulting in suppression of carbohydrate-specific antibody production.[9] Also, TACAs are non-site-specifically coupled to carrier proteins, leading to vaccine heterogeneity. Random coupling may also result in modification of important recognition epitomes on the carrier protein,[10] and conjugation chemistry is often difficult to control, leading to glycoproteins with ambiguities in structure and composition, which may affect the reproducibility of an immune response.

A recent report has provided convincing evidence for cases in which zwitterionic polysaccharides (ZPSs) invoke an MHCII-mediated T-cell response *in the absence of proteins*.[11] Kasper and co-workers[5b,12] have identified capsular polysaccharide structures that induce a CD4+ T-cell response known to modulate bacterial abscess formation. An unusual ZPS, PS A1 (**1**), consisting of a tetrasaccharide-core repeating unit (∼120 units) carrying an electrostatic charge character on adjacent monosaccharides, elicits an immune response similar to that for exogenous proteins.

In an attempt to overcome some of the current challenges with protein−carbohydrate vaccine development and examine a new direction, we elected to focus our attention on a strategy that would profit from the inherent MHCII-mediated immune activation by **1** and conjugate a known TACA, Tn hapten (**3**), in order to probe the immunogenicity and specificity for the development of a novel cancer immunotherapy. We sought to exploit an entirely carbohydrate immunogen to potentially overcome nonrelated peptide binding (cross reactivity with "self" proteins), generate carbohydrate-specific antibody production, and capitalize on a complete donor−acceptor sequence of carbohydrates for increased antibody−carbohydrate binding. Our strategy also incorporates a site-specific link between **1** and **3**, negating ambiguities in structure and composition.

Scheme 1. Synthesis of Tn−PS A1 Conjugate **4**



To garner appreciable amounts (∼500 mg) of **1**, a large-scale fermentation protocol employing the anaerobe *Bacterioides fragilis* NTCT 9343 was carried out.[13] Purified **1**, devoid of any protein and lipopolysaccharide, was subjected to selective oxidative cleavage conditions using 0.5 equiv of $NaIO_4$ in 0.10 M acetate buffer (pH 5.0) (Scheme 1). The oxidation protocol took advantage of the vicinal hydroxyl relationship (1° 6-OH, 2° 5-OH)[12] of the D-galactofuranose motif. The oxidation led to (hydrated) aldehyde **2**, providing a chemoselective handle. The addition of 5 equiv of KCl (to remove excess periodate as insoluble KIO_4)[13] followed by addition of *O*-2-NAc-D-Gal*p* hydroxylamine (**3**) afforded Tn−PS A1 oxime conjugate **4** after 18 h. The 500 MHz ^1H NMR spectrum of **4** in D_2O showed distinct oxime doublets of the *Z* and *E* isomers at 6.3 and 7.21 ppm, respectively.

With **1** and **4** in hand, we proceeded to test our hypothesis in 22 C57BL/6 mice. Mice were allowed to acclimate for a 1 week period, and prior to intraperitoneal (i.p.) immunizations, mouse sera were collected (day −1). The mice were divided into four subgroups consisting of eight, six, four, and four mice (groups A, B, C, and D respectively). **1** was administered i.p. to group A, **1** plus TiterMax Gold adjuvant to group B, **4** to group C, and **4** plus TiterMax Gold adjuvant to group D. The compounds were dissolved in PBS buffer (pH 7.4) at a concentration of 10 μg/0.1 mL. On days 0, 7, 14, 21, and 28, mice were immunized with 100 μL of the appropriate solution. On days 27 and 39, blood sera from groups A−D were collected and stored at −80 °C. All of the blood sera samples were then analyzed by enzyme-linked immunosorbent assay (ELISA).

PS A1−poly(L-lysine) and Tn−PS A1−poly(L-lysine) conjugates were synthesized using a well-known protocol[14] and used to coat

10.1021/ja902607a CCC: $40.75
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ELISA plates. Sera from groups A and B were used to determine the total antibody titer against **1** and the effect of the immune-stimulating adjuvant. The data in Table 1 indicate an ~200-fold increase in total antibody titer production specific for **1** relative to day −1, demonstrating a strong murine immune response. In fact, immunization with **1** + adjuvant did not show a dramatic increase in total antibody titer production relative to **1**, indicating that **1** can elicit an excellent immune response in the absence of adjuvant.

Table 1. Immunization Results with **1** and **4**

immunization[a]	ELISA coating[a]	goat anti-mouse kappa[a,b,c]					
		day −1[d]	day 39[d]	IgM[c,d]	IgG1[c,d]	IgG2a[c,d]	IgG3[c,d]
PS A1 (**1**)	**1**−poly(L-K)	0.5	101	−	−	−	−
1 + adjuvant	**1**−poly(L-K)	0.5	131	101	0[e]	0[e]	10
1 + adjuvant	**4**−poly(L-K)	−	−	60	0	0[e]	4
1 + adjuvant	**10**	−	−	2	0	0[e]	0
Tn−PS A1 (**4**)	**4**−poly(L-K)	0.4	110	−	−	−	−
4 + adjuvant	**1**−poly(L-K)	−	−	70	0	0[e]	5
4 + adjuvant	**4**−poly(L-K)	0.5	200	140	0[e]	0[e]	50
4 + adjuvant	**10**	0.5	99	77	0[e]	0[e]	6

[a] See the Supporting Information for details. [b] Specific for mouse κ light chains. [c] Titers were determined by linear regression analysis, plotting dilution versus absorbance. The highest dilution yielding an optical density (OD) of ≥0.2 relative to normal control mouse sera (day −1) was used to define the titers. [d] Value shown × 10^3. [e] Negligible amount.

In order to determine the specific antibody isotypes and provide further insight into the immune-processing pathway for PS A1 **1**, 2° antibodies to determine IgM and IgG isotypes IgG1, IgG2a, and IgG3 were used in an ELISA. The data for immunization **1** + adjuvant in Table 1 clearly illustrate that an abundance of IgG3 antibodies was produced, indicating a murine class switch carbohydrate-based immune response corresponding to a T-cell-dependent pathway.[4c,d]

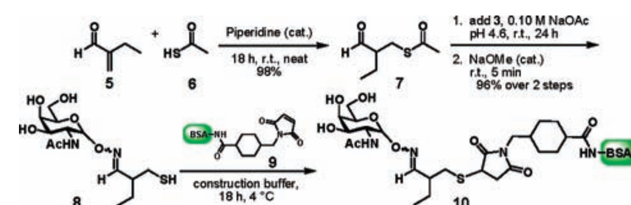
In an attempt to determine whether selective antibodies would arise against the Tn−PS A1 conjugate **4**, we examined blood sera from groups C and D. The data in Table 1 indicate a 200-fold increase (relative to day −1 sera) in total antibody titers produced against **4** in the absence of adjuvant, very similar to the result for the corresponding immunization with **1**. This implies that modification of **1** through the process of oxidation, yielding **2**, followed by oxime formation did not alter the ability to elicit an immune response. In order to confirm a T-cell-dependent immune response, we examined the specific antibody isotypes. The Table 1 data show an IgG3 response to **4** + adjuvant when **4**−poly(L-K) was used to coat the plate. This result implies that chemical modification of **1** did not alter the ability of **4** to target the T-cell-dependent pathway, most likely because the alternating charge character on adjacent monosaccharides was not changed.[12]

To further understand the specificity for conjugated Tn hapten (**3**), we designed an ELISA to determine whether IgG antibodies would bind to the hapten specifically. A crossover ELISA using sera from **1** + adjuvant with **4**−poly(L-K) as the coating and sera from **4** + adjuvant with **1**−poly(L-K) as the coating revealed that antibodies recognize PS A1 as a component of the desired immunogen.

The synthesis of conjugate with BSA tethered to Tn (**10**) (Scheme 2) commenced using 2-ethylacrolein (**5**) and thioacetic acid (**6**) to give **7** in 98% yield. Compound **7** was dissolved in a 0.1 M sodium acetate buffer (pH 4.6), after which **3** was added. Thioacetal deprotection gave **8** in near-quantitative yield. Compound **8** was immediately treated with BSA−maleimide **9** predissolved in construction buffer, and the mixture was stirred at 4 °C for 18 h. After purification and determination of the coupling efficiency, **10** was used to coat the ELISA plate.

Data from Table 1 for trials using **10** to coat the ELISA plate illustrate a high affinity of total antibody titer toward **3**. This result implies that the oxime link in Tn−PS A1 conjugate **4** did not undergo hydrolysis during immune processing and that anti-Tn antibodies were generated. 2° antibodies were used to determine the specificity of immunoglobulin binding toward **3**. The data show IgG3 specificity and imply a T-cell-dependent immune response.

Scheme 2. Synthesis of ELISA Plate Coating **10** for Determining Antibody Specificity against the Tn Hapten



In summary, we have designed, chemically synthesized, and immunologically evaluated an entirely carbohydrate vaccine candidate. We have demonstrated that chemical modification of zwitterionic polysaccharide PS A1 **1** does not alter or negate an immune response. Tn−PS A1 conjugate **4** can elicit exceptionally high titer antibodies even in the absence of an immune stimulant. Our design for a Tn−PS A1 conjugate takes advantage of site-specific conjugation to a modified D-galactofuranoside. ELISA results confirm immunoglobulin specificity toward Tn hapten **3**, which makes this a viable route for further exploration. We are currently pursuing the conjugation of other known TACAs and determining antibody specificity produced in sera as a measure for cross-reactivity.

Acknowledgment. Prof. Zhongwu Guo (WSU) is acknowledged for assistance with mouse studies. P.R.A. thanks WSU for start-up funds and a WSU Research Grant (145767).

Supporting Information Available: Experimental protocols, NMR spectra for all new compounds, and complete ref 7a. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA902607A