Glycoconjugate Journal (1998) **15:** 217–221

A novel and efficient method for synthetic carbohydrate conjugate vaccine preparation: synthesis of sialyl Tn-KLH conjugate using a 4-(4-*N*-maleimidomethyl) cyclohexane-1-carboxyl hydrazide (MMCCH) linker arm

Govindaswami Ragupathi[1]*, R. Rao Koganty[2], Dongxu Qiu[2], Kenneth O. Lloyd[3] and Philip O. Livingston[1]

[1] *Clinical Immunology Service, Memorial Hospital and* [3] *Immunology Program, Sloan-Kettering Institute, 1275 York Avenue, New York, NY-10021, USA*
[2] *Biomira, Inc., 2011-94 Street, Edmonton, Alberta, Canada T6N 1H1*

STn (NeuAcα2 → 6GalNAcα-O-Ser/Thr) is a carbohydrate epitope overexpressed in various human carcinomas. Clinical trials are underway using synthetic STn or STn trimeric glycopeptides [STn, cluster; STn(c)] conjugated with keyhole limpet hemocyanin (KLH) as active specific immunotherapy for these cancers. These vaccines have been prepared by conjugating a crotyl ethyl amide derivative of STn or STn(c) to KLH by direct reductive amination after ozonolysis. In the case of STn(c) the conjugation efficiency and the resulting epitope ratios were low. This may be due to steric hinderance of the short spacer arm. To overcome these difficulties, without resynthesis, the STn(c) glycopeptide was modified by attachment of an MMCCH (4-(4-*N*-maleimidomethyl) cyclohexane-1-carboxyl hydrazide) spacer arm to the aldehyde derivative, and then conjugated with thiolated KLH. This method gave a higher epitope ratio and yield than the direct method. The STn(c)-MMCCH-KLH conjugate induced high titer antibodies in mice against STn(c). This method may be generally applicable for large synthetic oligosaccharides.

***Keywords*: cancer, carbohydrate, conjugation, immunogen, vaccines**

Introduction

The purpose of conjugate vaccines against cancer is to instruct the immune system to recognize carbohydrate or peptide antigens expressed on the cancer cell surface. Many of these antigens are low molecular weight compounds or self antigens and hence poorly immunogenic by themselves. Such antigens can be made immunogenic by conjugating them with immunogenic carrier proteins such as tetanus or diphtheria toxins, bovine serum albumin (BSA), or keyhole limpet hemocyanin (KLH). Several studies with conjugate cancer vaccines containing natural or synthetic antigens have been reported [1–6]. One such example used conjugates of STn, a mucin-associated disaccharide (NeuAcα2 → 6GalNAcα-) O-linked to serine/threonine, which is expressed in many types of human adenocarcinomas, including carcinomas of the colon, breast, prostate, pancreas, ovary, stomach and lung. Expression on the corresponding normal tissues is described as limited or absent [7–11]. We have analyzed the cell surface STn configuration using sera from mice immunized with several STn-KLH conjugates and a panel of STn reactive monoclonal antibodies. The study revealed that STn is expressed at the tumor cell surface in at least two quite distinct configurations clustered and unclustered. This prompted us to develop an STn(c) based vaccine for breast cancer [12].

In this study monomeric STn-serine and clustered STn-serine trimeric glycopeptides were synthesized with crotyl linker arms and conjugated with KLH by ozonization followed by reductive amination. The epitope ratio of the STn-KLH conjugate was 3000:1, but the ratio for STn(c)-KLH conjugate was only 30:1 [12]. This may have been due to steric hindrance caused by the larger STn(c) molecule and reduced availability of the generated aldehyde for

* To whom correspondence should be addressed. Tel: (212)639-8554; Fax: (212)794-4352; E-mail: ragupatg@mskcc.org.

0282–0080

conjugation. To overcome these difficulties a more efficient method of conjugation utilizing 4-(4-*N*-maleimidomethyl) cyclohexane-1-carboxyl hydrazide (MMCCH) as a bridging group to 2-iminothiolane-derivitized KLH (thiolated KLH) has been developed. We demonstrate that the STn(c)-MMCCH-KLH conjugate had a higher epitope ratio, a better yield and induced higher titer antibodies against STn(c) than STn(c)-KLH prepared by the direct method.

Material and methods

Materials

STn(c) with a crotyl group (Compound 1) was synthesized by Biomira Inc., Edmonton, Alberta, Canada. Clinical grade KLH was purchased from PerImmune Inc., Rockville, MD. MMCCH (4-(4-*N*-maleimidomethyl) cyclohexane-1-carboxyl hydrazide HCl), 2-Iminothiolane (Trout's reagent), Ellman's reagents [5,5′-dithio-bis (2-nitrobenzoic acid)] and cysteine were purchased from Pierce, Rockford, IL.

CB6F1 female mice aged 6 weeks were obtained from Jackson Laboratory, Bar Harbor, ME.

Methods

Preparation of STn(c) aldehyde (see Figure 1)

Five mg of Compound 1 in methanol was stirred at − 78 °C in a dry-ice/ethanol bath and ozone gas (Delzone Traveler Model ZO-150 ozonator) was passed through the solution for 10 min under vigorous stirring. The excess of ozone was then displaced with nitrogen over a period of 10 min. Methylsulfide (100 µl) was added and the reaction mixture was stirred at room temperature for 2 h. The solvent was removed under a stream of nitrogen. The resulting white solid was used directly in the subsequent conjugation step.

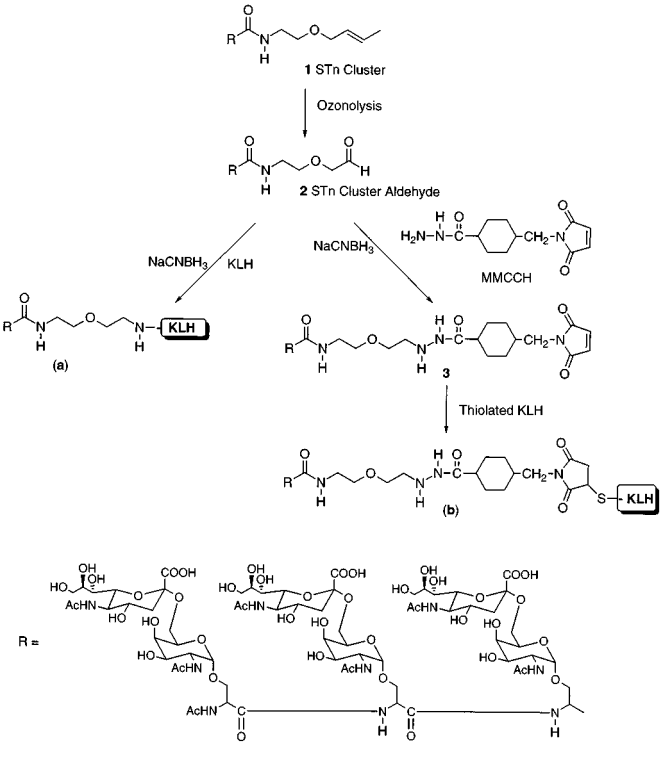

Figure 1. Synthesis of STn(c)-KLH conjugate after ozone cleavage of STn(c)-crotyl. (a) Direct method by reductive amination. (b) Crosslinker MMCCH method.

Direct conjugation of STn(c)-aldehyde with KLH

Five mg STn(c)-aldehyde (Compound 2) was dissolved in 1 ml of 0.1 M phosphate buffered saline (PBS), pH 7.2, and 15 mg of KLH in PBS. Five mg sodium cyanoborohydride was added and the mixture incubated under gentle agitation at 37 °C for 48 h. After 16 h, an additional 2.5 mg sodium cyanoborohydride was added and the incubation continued. The mixture was diafiltered using a Amicon Centriprep with molecular weight cut-off value 30 000 Da, with 6–7 changes of PBS at 4 °C. The presence of conjugate and absence of free STn(c) was checked by HPTLC with chloroform:methanol:water (60:40:10). STn(c)-KLH conjugate did not migrate in solvent but remained at the origin as a resorcinol-positive band [13].

Conjugation of STn(c)-aldehyde through MMCCH to thiolated KLH

Preparation of STn(c)-MMCCH. Five mg of STn(c)-aldehyde (Compound 2) was dissolved in 1 ml of 0.1 M sodium acetate buffer, pH 5.5, and 10 mg of MMCCH in 100 µl of dimethyl sulfoxide (DMSO) was added. The reaction mixture was incubated at room temperature for 15 min with gentle stirring. At the end of 15 min, 5 mg of solid sodium cyanoborohydride was added and the incubation continued at room temperature for 2 h. Unreacted MMCCH was removed in a Sephadex G10 column equilibrated previously with 0.1 M sodium phosphate buffer, pH 6.0, containing 5 mM EDTA and eluted with the same buffer. The fractions positive for STn(c) by HPTLC with resorcinol were combined.

Addition of sulfhydryl groups to KLH. 2-Iminothiolane (7.5 mg) dissolved in thiolation buffer (50 mM triethanolamine, 0.15 M NaCl, 5 mM EDTA, pH 8.0) was added to 15 mg of KLH and incubated with stirring at room temperature for 2 h. Unreacted 2-iminothiolane was removed by Sephadex G15 column equilibrated previously with 0.1 M sodium phosphate buffer, pH 7.2, containing 5 mM EDTA, and eluted with the same buffer. Fractions positive for KLH with BioRad protein assay dye reagent were combined. A small portion was used to estimate sulfhydryl groups in the thiolated KLH using Ellman's reagents and cysteine as standard as described earlier [14]. The KLH was estimated by a dye method using BioRad dye reagent according to the manufacturer's instructions.

Conjugation of STn(c)-MMCCH to thiolated KLH. The STn(c)-MMCCH product (Compound 3) and thiolated KLH were mixed and adjusted to pH 7.2 with 0.1 M sodium phosphate buffer, pH 8.0. The reaction mixture was then incubated at room temperature overnight. The content of the STn(c)-MMCCH-KLH reaction vial was transferred to a Centriprep concentrator 30 (Amicon: molecular cut-off 30 000 Da) and unreacted STn(c)-MMCCH was removed completely with multiple washes. The conjugate was checked by HPLTC for the absence of unreacted STn(c) as mentioned above. The epitope ratios of two batches of conjugate were determined by estimating sialic acid content using the resorcinol method described by Svennerholm [13] and protein content by the BioRad dye binding protein method as mentioned above.

Immunization of mice

Groups of mice (CB6F1 female; 6 weeks of age) were immunized subcutaneously with STn(c)-KLH or STn(c)-MMCCH-KLH containing 3 μg carbohydrate (the quantity of KLH varied depending on the epitope density) mixed with 10 μg of immunological adjuvant QS-21, a purified saponin fraction derived from the bark of the *Quillaja saponaria* Molina tree [15] (Aquila, Worcester, MA) at 0, 1 and 2 weeks and bled 10 days after the third immunization. The presence of antibody was assayed by an enzyme linked immunosorbent assay (ELISA) as described previously [12]. In brief, STn(c)-HSA or STn-HSA conjugates (prepared by Biomira by the direct amination method) were coated on ELISA plates at 0.1 μg (STn/STn(c)) per well in PBS. Serially diluted antiserum was incubated with the coated antigen for 1 h at room temperature and washed thrice in PBS containing 0.05% Tween 20. Adherent antigen-antibody complexes were then detected with goat anti-mouse IgG and IgM conjugated with alkaline phosphatase and p-nitrophenyl phosphatase as substrate. The ELISA titer was defined as the highest dilution yielding an absorbance of 0.10 or greater over that of normal sera.

Results and discussion

Initially we conjugated STn(c) glycopeptide (Compound 1) to KLH by the direct method. The yield and number of STn(c) per KLH were low (see Table 1) and did not provide sufficient STn(c) to explore immunogenicity in mice or patients. Consequently we devised a new conjugation method using the same starting compound. In this method Compound 1 was derivatized with MMCCH and the resulting compound was coupled to thiolated KLH (Figure 1). The amount of 2-iminothiolane required to maximally thiolate the KLH has first determined. The results (Table 2) showed that the most efficient ratio of KLH and 2-iminothiolane was 2:1 (w/w), which is equivalent to a mole ratio of 1:31 245 (assuming a MW of 8.6×10^6 for KLH). The thiolation of KLH is required because naturally KLH has only about 100 sulfhydryl groups (estimated with Ellman's reagent as described above) [14, 16]. This method consistently yields 1200–1300 sulfhydryl groups per KLH. Direct conjugation by reductive amination conjugated 30–100 mol STn(c) per mol KLH, while the MMCCH cross-linker method conjugated 368 to 464 mole STn(c) per mol KLH (Table 1). In addition, the yield with the MMCCH method was four times larger.

The antibody response following immunization with the STn(c)-KLH and STn(c)-MMCCH-KLH conjugates was tested by ELISA against STn(c)-HSA and STn-HSA as target antigens. The results are summarized in Table 3. Although there was some variation between individual mice we concluded: (i) both conjugates were immunogenic; (ii) IgG and IgM responses were obtained but the IgG responses were significantly higher in titer than the IgM responses; (iii) STn(c)-MMCCH-KLH conjugate induced significantly higher titer antibodies than the STn(c)-KLH conjugate; and (iv) unlike the antibody induced by STn(c)-KLH, the antibody induced by STn(c)-MMCCH-KLH cross reacted slightly with the monomer of STn.

Table 1. Preparation and analysis of STn(c)-KLH conjugates prepared by different methods

Expt. No.	*Amount used*		*Ratio of STn(c) KLH*	*STn(c) and KLH recovery (Wt)*		*Recovery (%)*		*Epitope ratio of conjugate STn(c)/KLH*
	STn(c) (mg)	*KLH (mg)*		*STn(c) (mg)*	*KLH (mg)*	*STn(c)*	*KLH*	
1	5.0	15	1:3	0.26	12.3	5.12	82.2	94.0
2	5.0	15	1:3	0.28	12.2	5.66	81.7	96.7
3	5.0	15	1:3	1.12	11.0	22.5	73.3	463.9
4	5.0	15	1:3	1.13	14.0	22.75	93.3	367.8

Experiments 1 and 2 are by direct reductive amination method. Experiments 3 and 4 are by cross-linker MMCCH method.

Table 2. Incorporation of sulfhydryl groups in KLH by different mole ratios of 2-iminothiolane.

Experiment	*KLH (mg)*	*2-iminothiolane (mg)*	*Mole ratio KLH:2-imino*	*Number SH/KLH*[a]
1	3	0.45	1:9373	1001
2	3	1.5	1:31 245	1251
3	3	3.0	1:62 491	1287

[a] Average values of two different experiments.

Table 3. Anti-STn(c) and anti-STn antibody titers by ELISA against STn(c)-HSA and STn-HSA conjugates

Mouse	*STn(c)-HSA*		*STn-HSA*	
	IgM	*IgG*	*IgM*	*IgG*
3 μg STn(c)-KLH + 10 μg QS-21 per mouse				
1–1	450	1350	50	0
1–2	150	800	50	0
1–3	150	4050	0	0
1–4	50	1350	0	0
1–5	0	0	0	0
Median	150	1350	0	0
3 μg STn(c)-MMCCH-KLH + 10 μg QS-21 per mouse				
2–1	150	4050	50	150
2–2	1350	1350	150	50
2–3	4050	109 350	1350	1350
2–4	12 150	204 800	4050	1350
2–5	12 150	109 350	50	50
Median	4050	109 350	150	150

Value 0 is < 50 dilution.

The approach of using protein conjugates to improve the immunogenicity of small molecular weight haptenic molecules has been well known since the early work of Landsteiner [17]. Landsteiner also determined the optimal number of haptenic groups per carrier (epitope ratio) and concluded that too much or too little hapten led to a lower antibody response against the hapten. More recent studies using BSA as a carrier protein with different epitode ratios have yielded the same result [18]. No such studies have been previously reported with KLH, though it is used widely as a carrier protein in human immunotherapy; in this study we show that a conjugate with a higher epitose ratio is more immunogenic.

Procedures for the preparation of conjugates vary depending on the functional group(s) present on the antigen and there is no method which is universally applicable. Different methods result in different epitope ratios, different yields, and different immunogenicities of the resulting conjugates [19, 20]. In our hands, especially when used as a vaccine in humans, KLH is the preferred carrier protein for conjugation because of its high molecular weight, high immunogenicity and the many lysine groups available for conjugation [2, 5, 6, 12, 16, 21]. We have extensive experience in conjugating gangliosides like GM2, GD2 and GD3 to KLH by the reductive amination method with epitope ratios between 500–1000 generally resulting [5, 21, 22]. When we conjugated STn(c) by direct reductive amination, only 30–100 STn(c) haptens were conjugated per KLH. This may have been due to steric hindrance caused by the large STn(c). To over come this problem we have introduced an MMCCH spacer arm using the bifunctional cross-linker molecule MMCCH. The STn(c)-MMCCH-KLH conjugate gave a higher epitope density than was possible with STn(c)-KLH without the spacer arm. When the two conjugates were used to immunize mice, the STn(c)-MMCCH-KLH conjugate was found to elicit a higher titer antibody response than STn(c)-KLH. The higher antibody titer is probably due to the higher epitope ratio but may also be due to qualitative issues related to the carbohydrate epitopes available to the immune system. Enhanced antibody titers have previously been reported with different haptens when spacer arms were introduced between carrier molecule and antigen [23–25]. Different and improved specificity of induced antibodies has also been reported with conjugates using linkers [23–25].

It has been our previous experience that the nature of the antigen, the carrier protein, the location on the antigen selected for conjugation to the carrier and the adjuvant all are important in obtaining an optimal antibody response [21]. We report here that the method of conjugation is also important. With STn(c) as antigen, using a MMCCH linker arm resulted in a higher epitope ratio, a higher yield and improved IgM and IgG antibody responses compared to STn(c)-KLH prepared without this linker arm. On the basis of these studies, a phase I trial with STn(c)-MMCCH-KLH plus QS-21 in patients with breast cancer has been initiated.

Acknowledgement

This work is supported by grants from the National Institute of Health (CA 61422, CA 71506 and CA 08748), CapCure and the Pepsico fund.

References

1 O'Boyle KP, Zamore R, Adluri S, Cohen A, Kemeny N, Welt S, Lloyd KO, Oettgen HF, Old LJ, Livingston PO (1992) *Cancer Res* **52**: 5663–7.

2 Adluri S, Helling F, Ogata S, Zhang S, Itzkowitz SH, Lloyd KO, Livingston PO (1995) *Cancer Immunol Immunother* **41**: 185–92.

3 MacLean GD, Reddish M, Koganty RR, Wong T, Gandhi S, Smolenski M, Samuel J, Nabholtz JM, Longenecker BM (1993) *Cancer Immunol Immunother* **36**: 215–22.
4 Longenecker BM, Reddish M, Miles D, MacLean GD (1993) *Vaccine Res* **2**: 151–62.
5 Ragupathi G (1996) *Cancer Immunol Immunother* **43**: 152–57.
6 Ragupathi G, Park TK, Zhang S, Kim IJ, Graber L, Adluri S, Lloyd KO, Danishefsky SJ, Livingston PO (1997) *Angew Chem Int Ed Engl* **36**: 125–8.
7 Itzkowitz SH, Bloom EJ, Kokal WA, Modin G, Hakomori S, Kim YS (1990) *Cancer* **66**: 1960–6.
8 Itzkowitz SH, Kjeldsen T, Friera A, Hakomori S, Yang U, Kim YS (1991) *Gastroenterology* **100**: 1691–700.
9 Werther JL, Rivera-MacMurry S, Bruckner HB, Tatematsu M, Itzkowitz SH (1994) *Br J Cancer* **69**: 613–16.
10 Thor A, Ohuchi N, Szpak CA, Johnson WW, Schlom J (1986) *Cancer Res* **46**: 3118–24.
11 Xu M, Real FX, Welt S, Schussler MH, Oettgen HF, Old LJ (1989) *Int J Cancer* **44**: 985–9.
12 Zhang S, Walberg LA, Ogata S, Itzkowitz SH, Koganty RR, Reddish M, Gandhi SS, Longenecker MB, Lloyd KO, Livingston PO (1995) *Cancer Res* **55**: 3364–8.
13 Svennerholm L (1957) *Biochem Biophys Acta* **24**: 604–11.
14 Riddles PW, Blackeley RL, Zerner B (1979) *Anal Biochem* **94**: 75–81.
15 Kensil CR, Patel U, Lennick M, Marciani D (1991) *J Immunol* **146**: 431–7.
16 Swerdlow RD, Ebert RF, Lee P, Bonaventura C, Miller KI (1996) *Comp Biochem Physiol* **113B**: 537–48.
17 Landsteiner K (1945) In *The Specificity of Serological Reactions*. Cambridge, Massachusetts: Harvard University Press.
18 Klaus GG, Cross AM (1974) *Cell Immunol* **14**: 226–41.
19 Williams DG (1986) In *Immunoassay Technology*, (Pal SB, ed.) Vol 2, pp 1–15 Berlin: Walter de Gruyter & Co.
20 Erlanger BF (1973) *Pharmacol Rev* **25**: 271–80.
21 Helling F, Shang A, Claves M, Zhang S, Ren S, Yu RK, Oettgen HF, Livingston PO (1994) *Cancer Res* **54**: 197–203.
22 Helling F, Zhang S, Shang A, Adluri S, Calves M, Koganty R, Longenecker BM, Yao TJ, Oettgen HF, Livingston PO (1995) *Cancer Res* **55**: 2783–8.
23 Lindner HR, Perel E, Friedlander A, Zeitlin A (1972) *Steroids* **19**: 357–75.
24 Exley D, Johnson MW, Dean PDG (1971) *Steroids* **18**: 605–20.
25 Jeffcoate SL, Searle JE (1972) *Steroids* **19**: 181–8.

Received 23 April 1997, revised 4 July 1997, accepted 11 July 1997