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Vaccines prepared with sialyl-Tn and sialyl-Tn trimers using the 4-(4-maleimidomethyl)cyclohexane-1-carboxyl hydrazide linker group result in optimal antibody titers against ovine submaxillary mucin and sialyl-Tn-positive tumor cells

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Abstract Sialyl-Tn (STn) is an *O*-serine- or *O*-threonine-linked disaccharide [NeuAcα(2→6)GalNAcα-*O*-Ser/Thr) expressed on mucins of most types of adenocarcinoma as single STn or clustered STn [STn (c)] epitopes. Though STn is expressed on some normal tissues it is relatively tumor-specific, especially in the clustered conformation. Clinical trials with STn-keyhole limpet hemocyanin (KLH) conjugate vaccines, prepared using reductive amination with a two-carbon linker group, have resulted in high titers against STn but lower titers against natural forms of STn (ovine submaxillary mucin, or tumor cells). To obtain antibodies of more appropriate specificity, we attempted to prepare STn(c)-KLH conjugates to establish their immunogenicity in mice in preparation for clinical trials; however, conjugation efficiency was poor when the same two-carbon linker was used, presumably because of steric hindrance. STn-KLH and STn(c)-KLH conjugates were prepared using the regular two-carbon or the recently developed more efficient longer heterobifunctional 4-(4-maleimidomethyl)cyclohexane-1-carboxyl hydrazide (MMCCH) linkers, and the resulting immunogenicities in mice were compared. The highest titers against STn were seen with the STn-KLH conjugate with the two-carbon linker, and the highest titers against STn(c) were seen with STn(c)-KLH with the MMCCH linker. Conjugation with MMCCH resulted in the highest conjugation efficiency (yield) and the highest titers against ovine submaxillary mucin and STn-positive tumor cells, and is the method of choice for the preparation of STn(c) vaccine for clinical trials.

Key words Carbohydrate · Conjugation · Immunogen · Immunotherapy · Sialyl Tn · Vaccine

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Introduction

Sialylated Tn [STn, NeuAcα(2→6)GalNAcα-*O*-Ser/Thr] is a mucin-associated carbohydrate antigen *O*-linked to serine or threonine of mucins expressed on a variety of epithelial cancers. More than 80% of cancers of breast, prostate and ovarian origin express STn with no or limited expression on the corresponding normal tissues [10, 11, 24, 34, 43, 46]. STn expression by various epithelial cancers correlates with a more aggressive phenotype and a more ominous prognosis [43]. STn is the epitope recognized by monoclonal antibody B72.3 and probably by monoclonal antibody CC49 [46], two antibodies that have been widely used for diagnostic and therapeutic purposes [6, 29, 39, 40]. STn expression on normal tissues, defined by B72.3, is restricted to a few epithelial tissues at secretary borders. Immunization with STn has been shown to induce anti-STn antibodies and to protect mice from subsequent challenge with syngeneic cancer cell lines expressing STn [5]. Hence both active and passive immunotherapy studies have identified STn as a uniquely effective target for antibody-mediated cancer immunotherapy. This is consistent with an expanding body of data demonstrating the ability of antibodies against defined tumor antigens to protect against circulating tumor cells and micrometastases (reviewed in [20, 22]). These points form the basis for the construction of cancer vaccines against STn designed to be used in the clinic in the adjuvant setting.

Patients with a variety of epithelial cancers have been immunized with STn monomer conjugated to the immunogenic carrier protein keyhole limpet hemocyanin (KLH) plus various adjuvants [1, 25–28, 32]. High-titer IgM and IgG antibodies against STn have resulted. Surprisingly, in some cases, much of this reactivity has been against antigenic epitopes present mainly on the synthetic constructs. The IgM antibodies reacted with moderate titer against natural mucins or tumor cells expressing STn, and the IgG antibodies were less reactive [1]. On the basis of previous studies with Tn antigen [16], Nakada et al. hypothesized that MLS128, a monoclonal antibody against STn, which reacted more strongly with ovine submaxillary mucin (OSM) than with single STn epitopes, might preferentially recognize clusters of STn [31]. Our studies with monoclonal antibody B72.3 and with sera raised against STn-KLH conjugate vaccines in mice and patients resulted in the same conclusion [1, 46]. The availability of synthetic STn trimers or clusters [STn(c); see Fig. 1] permitted us to prove this hypothesis. In both direct tests and inhibition assays, B72.3 recognized exclusively STn clusters and reacted strongly with both natural mucins (OSM) and tumor cells expressing STn [46]. These results demonstrated that STn is recognized at the tumor cell surface in at least two quite distinct configurations, and that the remarkable specificity for tumor cells shown by mAb B72.3 may be a consequence of its specificity for clustered STn [46]. We propose to test an STn(c) vaccine in patients with STn-positive cancers. Recently we developed a more efficient method for conjugating STn or STn(c) to KLH using a bifunctional linker group [4-(maleimidomethyl)cyclohexane-1-carboxyl hydrazide, MMCCH] and thiolated KLH [36]. We compare here the immunogenicity in mice of STn and STn(c) conjugated to KLH by the older direct method and by the newer MMCCH method and demonstrate that the resulting antibodies react strongly with OSM and tumor cells expressing STn.

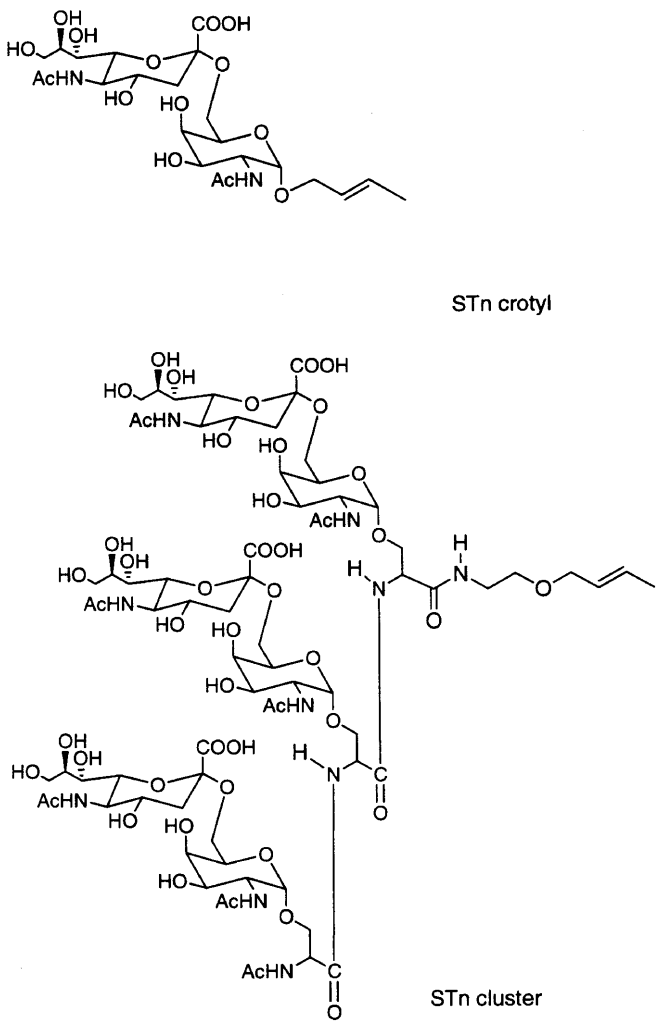

Fig. 1 Structure of the crotyl derivatives of sialyl-Tn (STn) and STn cluster

Materials and methods

Antigens and adjuvants

Crotyl-STn, crotyl-STn(c) (Fig. 1), STn linked to human serum albumin (STn-HSA) and STn(c)-HSA were synthesized by Biomira Inc., (Edmonton, Alberta, Canada). STn(c) is synthesized with three STn disaccharides attached to three serines as shown in Fig. 1. Clinical-grade KLH was purchased from PerImmune Inc., (Rockville, Md.). Cross-linker MMCCH, the thiolation reagent 2-iminothiolane (Trout's reagent), Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)] for free sulfhydryl determination, and cysteine were purchased from Pierce (Rockford, Ill.). OSM was purchased from BioCarb (Lund, Sweden). Adjuvant QS-21, containing a Quill A saponin fraction purified from the bark of the *Quillaja saponaria* Molina tree [13], was obtained from Aquila Biopharmaceuticals (Worcester, Mass.).

Vaccine preparations

STn-KLH and STn(c)-KLH conjugates were prepared by two methods, a direct reductive amination method as described earlier [30, 38] and with an MMCCH linker arm [38]. The STn-MMCCH-KLH and STn(c)-MMCCH-KLH conjugates were prepared by a recently developed procedure [38]. In brief, an aldehyde group was generated by ozonolysis of the crotyl derivative of STn or STn(c) and this was coupled to the hydrazide group of MMCCH. The maleimide group of MMCCH was then reacted with KLH, which had been thiolated by treatment with 2-iminothiolane, which converts NH_2 groups in KLH to sulfhydryl groups; the free sulfhydryl groups were determined by Ellman's reagents with cysteine as standard. The reaction was continued at room temperature for 4 h. The unreacted STn or STn(c) was removed with the help of a Centriprep concentrator 30 (Amicon, molecular cut-off 30 kDa). The epitope ratios were determined by estimating sialic acid content using the resorcinol method described by Svennerholm [42] and KLH content by the BioRad dye-binding method according to the manufacturer's instructions. The vaccines used, the conjugated epitope densities achieved, and the overall yield for STn and the STn cluster are summarized in Table 1.

Animals and vaccinations

Groups of five mice (CB6F1; female, 6 weeks of age) obtained from Jackson Laboratory (Bar Harbor, Me.) were subcutaneously immunized with 3 μg STn or STn(c), unconjugated STn mixed with 20 μg KLH, or STn(c) mixed with 20 μg KLH or conjugated to

KLH by two methods (the quantity of KLH varied, depending on the epitope density), plus 10 μg immunological adjuvant QS-21. Mice were injected on days 0, 7 and 14 and bled 10 days after the 3rd immunization. Two more booster injections were given 10 and 19 weeks after the first vaccination. Mice were bled before the booster and 10 days after each booster injection.

Enzyme-linked immunosorbent assay (ELISA) antibody titers against STn-HSA, STn(c) HSA and OSM

ELISA were performed as described previously [1]. For testing, the ELISA plates were coated with 0.1 μg/well STn-HSA, STn(c)-HSA or OSM. Conjugates prepared with MMCCH were not tested. Serially diluted antisera were incubated in coated wells for 1 h at room temperature. Goat anti-(mouse IgG) or IgM alkaline-phosphatase-labeled secondary antibodies at a dilution of 1:200 (Zymed, San Francisco, Calif.) were used. The titer was defined as the highest dilution yielding an absorbance of 0.10 or greater over that of mouse serum drawn prior to the initial vaccination.

Table 1 Conjugates used and their epitope densities. *STn* sialyl-Tn, *KLH* kehole limpet hemocyanin, *MMCCH* 4-(maleimidomethyl) cyclohexane-1-carboxyl hydrozide, *STn*(*c*) STn cluster (three STn epitopes). Calculations were based on the following molecular masses: KLH 8.6 MDa, crotyl-STn 554 Da, crotyl-STn(c) 1900 Da

Conjugate	Number of epitopes/ KLH (mol/mol)	STn or STn(c) conjugated with KLH (yield, %)
STn-KLH	394	7.2
STn-MMCCH-KLH	1123	21.7
STn(c)-KLH	95.4	5.4
STn(c)-MMCCH-KLH	409	22.6

Cell surface reactivity determined by fluorescence-activated cell sorting (FACS) assay

FACS was performed as previously described [45]. The cell-surface assay reactivity of anti-STn and anti-STn(c) antibodies was tested on LS-C (STn-positive) and LS-B (STn-negative) cells provided by Dr. S. H. Itzkowitz [33]. Single-cell suspensions of 2×10^5 cells/tube were washed in phosphate-buffered saline (PBS) with 3% fetal calf serum (FCS) and 0.01 M NaN_3 and incubated with 20 μl 1:20 diluted antisera or monoclonal antibody B72.3 (provided by Dr. Schlom, NCI) for 30 min on ice. After two washes with 3% FCS in PBS, 20 μl 1:15 diluted goat anti-(mouse IgM) or anti-(IgG) labeled with fluorescein isothiocyanate was added, and the mixture was incubated for 30 min. After a final wash, the positive population and mean fluorescence intensity of stained cells were differentiated using an EPICS Profile II flow cytometer (Coulter, Co., Hialeah, Fler.).

Results

Antibody response against STn monomer

ELISA antibody titers of sera from mice immunized with KLH conjugated to STn, STn(c), STn-MMCCH

Table 2 Enzyme-linked immunosorbent assay (ELISA) antibody titers against STn linked to human serum albumin (HSA)

Vaccine	Antibody titer before and after vaccination											
	Before		After 3rd		Before 4th		After 4th		Before 5th		After 5th	
	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
STn-KLH												
1-1[a]	0	0	4050	12 150	150	1350	1350	109 350	100	300	300	24 300
1-2	0	0	1350	450	50	4050	1350	109 350	100	2700	300	24 300
1-3	0	0	0	1350	50	1350	12 150	109 350	100	2700	100	24 300
1-4	0	0	1350	12 150	150	450	1350	109 350	100	2700	300	24 300
1-5	0	0	0	12 800	50	450	50	109 350	100	2700	0	8100
Median	0	0	1350	12 150	150	1350	1350	109 350	100	2700	300	24 300
STn-MMCCH-KLH												
2-1	0	0	150	4050	50	50	450	450	100	300	100	300
2-2	0	0	1350	12 150	0	0	50	1350	300	100	300	300
2-3	0	0	150	4050	0	50	50	450	100	100	900	100
2-4	0	0	150	4050	0	50	150	1350	100	100	100	900
2-5	0	0	450	150	0	50	150	450	0	0	100	100
Median	0	0	150	4050	0	50	150	450	100	100	100	300
STn(c)-KLH												
3-1	0	0	50	0	0	0	50	0	0	0	0	0
3-2	0	0	50	0	0	50	50	0	0	0	0	0
3-3	0	0	0	0	0	0	50	50	0	0	0	0
3-4	0	0	0	0	0	0	50	0	0	0	0	0
3-5	0	0	0	0	0	0	50	0	0	0	0	0
Median	0	0	0	0	0	0	50	0	0	0	0	0
STn(c)-MMCCH-KLH												
4-1	0	0	50	150	150	450	0	0	100	0	0	0
4-2	0	0	150	50	0	0	0	0	100	0	0	0
4-3	0	0	1350	1350	0	0	0	450	0	0	0	0
4-4	0	0	4050	1350	50	150	0	1350	100	100	100	0
4-5	0	0	50	50	0	50	0	150	100	0	Died	Died
Median	0	0	150	150	0	50	0	150	100	0	0	0

[a] Each number refers the result obtained from an individual mouse

and STn(c)-MMCCH were determined with synthetic STn monomer conjugated through the crotyl group directly to HSA. The results are summarized in Table 2. No detectable anti-STn IgM or IgG antibodies were present in sera obtained before vaccination (Table 2) or in control groups vaccinated with 3 μg STn plus 10 μg QS-21, 3 μg STn(c) plus 10 μg QS-21, 3 μg STn mixed with 20 μg KLH plus 10 μg QS-21, 3 μg STn(c) mixed with 20 μg KLH plus 10 μg QS-21 (data not shown). Sera obtained after vaccination with monomer STn conjugated to KLH directly or via MMCCH were able to react with STn monomer by ELISA but the titers were highest at all times points after vaccination with the direct conjugates. This was not unexpected since the STn-HSA target was also prepared by the direct conjugation method. Sera obtained by vaccination with STn(c)-KLH prepared by the direct method failed to react with STn monomer conjugates, while sera obtained by vaccination with STn(c)-MMCCH-KLH reacted slightly.

Antibody response against STn cluster

Antibody titers in the immunized mice against the synthetic STn(c) epitopes conjugated to HSA by direct reductive amination were determined by ELISA (Table 3). No detectable anti-STn(c) IgM or IgG antibodies were present in sera drawn prior to vaccination or after vaccination in control groups. Sera obtained after vaccination with STn monomers conjugated by the direct or cross-linker methods showed low reactivity with STn(c) after the initial four vaccinations. After the fifth vaccination with STn-KLH, strong IgG reactivity was present. On the other hand, sera obtained after three or more vaccinations with STn(c) conjugates prepared by direct or MMCCH methods reacted strongly with STn(c)-HSA. Titers with sera from mice immunized with STn(c)-MMCCH-KLH were induced more rapidly and subsequently were as high or higher than the titers of sera from mice in other groups.

Antibody response against natural antigens

The STn and STn(c) epitopes on OSM and LS-C colon cancer cells served as natural STn epitope targets for testing the antibody responses. The results of the tests on OSM are summarized in Table 4. No reactivity against OSM was seen in prevaccination sera or in the control groups. Sera obtained after vaccination with all four vaccines showed strong reactivity against OSM, the re-

Table 3 ELISA antibody titers against STn(c)-HSA

Vaccine	Antibody titer before and after vaccination											
	Before		After 3rd		Before 4th		After 4th		Before 5th		After 5th	
	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
STn-KLH												
1-1[a]	0	0	450	50	150	50	450	12 150	300	300	900	24 300
1-2	0	0	50	0	150	0	150	12 150	300	900	300	24 300
1-3	0	0	0	0	50	0	450	450	100	300	100	900
1-4	0	0	150	0	150	0	0	150	300	900	900	8100
1-5	0	0	0	0	150	0	0	450	300	900	300	24 300
Median	0	0	0	0	150	0	150	450	300	900	300	24 300
STn-MMCCH-KLH												
2-1	0	0	150	50	50	0	0	0	100	300	300	0
2-2	0	0	50	50	50	0	0	450	300	300	100	100
2-3	0	0	150	450	50	150	0	450	300	900	900	100
2-4	0	0	50	0	150	450	0	450	100	300	100	100
2-5	0	0	50	450	50	1350	0	0	100	100	300	0
Median	0	0	50	50	50	150	0	450	100	300	300	100
STn(c)-KLH												
3-1	0	0	450	1350	150	150	0	109 350	300	8100	300	72 900
3-2	0	0	150	800	50	450	0	109 350	100	900	100	24 300
3-3	0	0	150	4050	150	4050	0	109 350	100	8100	100	72 900
3-4	0	0	50	1350	50	150	0	109 350	300	2700	300	24 300
3-5	0	0	0	0	50	0	0	109 350	100	0	100	0
Median	0	0	150	1350	50	150	0	109 350	100	2700	100	24 300
STn(c)-MMCCH-KLH												
4-1	0	0	150	4050	50	1350	1350	4050	300	2700	100	24 300
4-2	0	0	1350	1350	50	50	4050	450	900	8100	100	8100
4-3	0	0	4050	109 350	50	450	150	109 350	300	8100	100	72 900
4-4	0	0	12 150	204 800	450	4050	12 150	109 350	2700	8100	300	218 700
4-5	0	0	12 150	109 350	50	1350	12 150	109 350	300	0	Died	Died
Median	0	0	4050	109 350	50	1350	4050	109 350	300	8100	100	24 300

[a] Each number refers the result obtained from an individual mouse

Table 4 ELISA antibody titers against ovine submaxillary mucin

Vaccine	Antibody titer before and after vaccination											
	Before		After 3rd		Before 4th		After 4th		Before 5th		After 5th	
	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
STn-KLH												
1-1[a]	0	0	4050	109 350	900	24 300	900	218 700	100	300	900	24 300
1-2	0	0	450	150	300	24 300	900	218 700	100	8100	900	72 900
1-3	0	0	300	450	100	2700	2700	218 700	100	900	300	8100
1-4	0	0	1350	109 350	100	24 300	900	218 700	100	2700	900	72 900
1-5	0	0	300	4050	100	2700	100	72 900	300	900	300	8100
Median	0	0	450	4050	100	24 300	900	218 700	100	900	900	24 300
STn-MMCCH-KLH												
2-1	0	0	450	36 450	100	72 900	8100	72 900	100	100	100	300
2-2	0	0	4050	109 350	100	72 900	900	72 900	300	300	300	900
2-3	0	0	1350	109 350	2700	72 900	900	72 900	300	300	900	300
2-4	0	0	450	12 150	300	72 900	900	24 300	100	300	100	900
2-5	0	0	450	1350	100	72 900	300	72 900	100	0	300	100
Median	0	0	450	36 450	100	72 900	900	72 900	100	300	300	300
STn(c)-KLH												
3-1	0	0	450	450	100	900	300	24 300	100	2700	300	24 300
3-2	0	0	150	4050	0	2700	300	24 300	0	2700	100	8100
3-3	0	0	450	1350	100	8100	300	72 900	0	8100	100	24 300
3-4	0	0	150	109 350	0	0	900	24 300	100	2700	300	24 300
3-5	0	0	50	50	0	0	100	0	100	0	2700	100
Median	0	0	150	1350	0	900	300	24 300	100	2700	300	24 300
STn(c)-MMCCH-KLH												
4-1	0	0	450	109 350	900	8100	72 900	24 300	100	2700	300	24 300
4-2	0	0	4050	109 350	2700	72 900	24 300	218 700	300	2700	900	72 900
4-3	0	0	12 150	109 350	8100	2700	24 300	218 700	300	24 300	300	218 700
4-4	0	0	4050	109 350	2700	72 900	72 900	218 700	900	24 300	900	24 300
4-5	0	0	4050	109 350	2700	24 300	72 900	72 900	300	0	Died	Died
Median	0	0	4050	109 350	2700	24 300	72 900	218 700	300	2700	300	49 000

[a] Each number refers the result obtained from an individual mouse

activity generally being highest in the STn(c)-MMCCH-KLH group.

Cell-surface reactivity of sera drawn 10 days after the 3rd and 4th vaccinations with STn and STn(c) vaccines were tested by flow cytometry using STn- and STn(c)-positive LS-C cells and STn- and STn(c)-negative LS-B cells. The results are summarized in Table 5. Sera from unimmunized mice showed minimal reactivity (less than 10% positive cells). After both the 3rd and 4th vaccinations, sera from mice vaccinated with all four vaccines showed clear IgM and IgG reactivity with LS-C cells by flow cytometry and low reactivity (7%–13% positive cells) against LS-B cells. Antibodies induced by conjugates prepared with the MMCCH cross-linker generally showed the highest level of cell-surface reactivity.

Discussion

Landsteiner first introduced the concept of hapten-protein conjugation to induce immune responses against nonimmunogenic compounds in 1917 [17]. Since then conjugation of nonimmunogenic or poorly immunogenic antigens with highly immunogenic foreign protein carriers has been applied to induce immune responses against a variety of antigens, including the widespread use of bacterial polysaccharides in humans [2, 4, 12, 41]. These vaccines result in high titers of antibodies, which present serious infections by eliminating circulating pathogens.

Treatment of cancer patients in an adjuvant setting shares similar goals, that is, elimination of micrometastases and circulating tumor cells. Though conjugate cancer vaccines have entered clinical trials [9, 18, 19, 21, 26, 35, 36], vaccine construction can be complex and many variables have yet to be addressed. The basic work of Landsteiner concluded that too much or to little hapten led to a lower antibody response against the hapten. Studies with bovine serum albumin (BSA) as carrier protein and different antigen/BSA epitope ratios have resulted in the same conclusions [14, 44]. No such studies have been previously reported with KLH, though it is the most widely used carrier protein in cancer immunotherapy. There is no single conjugation method that is universally applicable to conjugate low-molecular-mass compounds to KLH. The optimal conjugation methods vary, depending on the functional groups available, on the antigen, and on their location in relation to the antigenic epitope. Different methods result in different epitope ratios, different yields, and different immunogenicities of the resulting conjugates

Table 5 Fluorescence-activated cell sorting (FACS) analysis of anti-STn and STn(c) antibodies on LS-C (STn-positive) and LS-B (STn-negative) cells. [a] Presera and postsera obtained from mice bled 10 days after 3rd and 4th vaccination were used for FACS analysis on both LS-C and LS-B cell lines. Presera showed less than 10% positive on both LS-C and LS-B cell lines. Postsera showed low reactivity (7%–13% positive cells) on LS-B. Monoclonal antibody B72.3 (IgG1) showed 91.5% positive cells on LS-C and 1.46% positive cells on LS-B

Vaccine	Positive cells on LS-C cells (%)			
	10 days after 3rd vaccine		10 days after 4th vaccine	
	IgM	IgG	IgM	IgG
STn-KLH				
1-1[a]	37.1	27.4	80.76	97.59
1-2	18.9	11.1	61.51	96.29
1-3	15.5	18.6	96.00	92.85
1-4	46.3	25.9	92.23	95.95
1-5	21.2	20.8	21.54	85.27
Median	21.2	20.8	80.76	95.95
STn-MMCCH-KLH				
2-1	12.0	34.8	97.00	74.50
2-2	36.5	44.7	91.36	28.00
2-3	37.3	65.4	89.30	71.94
2-4	18.3	64.5	84.39	47.11
2-5	24.5	17.2	65.59	59.50
Median	24.5	44.7	89.30	59.50
STn(c)-KLH				
3-1	35.4	10.2	83.23	91.75
3-2	31.4	24.4	69.44	92.47
3-3	24.4	14.5	88.72	95.56
3-4	12.4	29.5	73.97	92.38
3-5	5.5	6.1	58.60	2.38
Median	24.4	14.5	83.23	92.47
STn(c)-MMCCH-KLH				
4-1	40.7	24.5	89.99	95.73
4-2	56.6	24.7	97.61	98.59
4-3	18.5	59.0	84.56	98.17
4-4	56.3	62.0	95.25	89.87
4-5	34.8	31.9	82.41	90.89
Median	40.7	31.9	89.99	95.73

[3, 8, 15, 37, 38]. In this study we show that the epitope ratio and the method of conjugation, in addition to the nature of the antigen, influence immunogenicity.

For this study, we endeavored to conjugate STn(c) with KLH by the standard direct reductive amination method. Our yields were low (generally around 5%), presumably because of steric hindrance by STn(c), making this approach to vaccine construction impractical. Recently we described a more efficient method for conjugating STn(c) to KLH using the bifunctional linker group in MMCCH [38]. We explore here the conjugation efficiency and immunogenicity of STn and STn(c) conjugated by the direct and MMCCH methods. Sera from mice immunized with STn-KLH reacted strongly with STn-HSA and weakly with STn(c)-HSA. On the other hand, sera from mice immunized with STn(c)-KLH conjugated by direct or MMCCH methods reacted strongly with STn(c)-HSA but weakly or not at all with STn-HSA. Each immunogen induced antibodies that react preferentially against the immunogen itself, though both induce antibodies that react with LS-C tumor cells. While OSM and LS-C tumor cells express both STn and STn(c), it is our opinion that STn(c) is a more tumor-specific antigen than STn, on the basis of studies with B72.3, which recognizes STn(c) [46] and reacts strongly with a variety of tumors but few normal cells [47]. Titers against STn(c)-HSA were generally highest when the MMCCH method was used for vaccine construction. This was especially significant since (i) the STn(c)-HSA target was conjugated by the direct method and (ii) the yields for STn(c)-KLH conjugation by the MMCCH method were consistently four- to fivefold greater than with the direct method.

The nature of the tumor antigen (the precise epitope on tumor cells recognized by the immune system) must be faithfully replicated in the vaccine for antibodies of appropriate specificity to be generated. In this study in mice, both STn-KLH and STn(c)-KLH induced high-titer antibodies against OSM and tumor cells expressing STn. These antibodies recognize STn on OSM and LS-C tumor cells in two different conformations, clustered or unclustered. In earlier studies in humans, immunization with STn-KLH and Detox adjuvant in breast, colorectal and ovarian patients produced both IgM and IgG antibodies reacting with OSM [27, 28]. In a separate study [1], immunization with STn-KLH with Detox or QS-21 resulted in weak to moderate IgM reactivity, and only low IgG reactivity with OSM. Our premise that clustered STn groups might be a more effective immunogen than STn monomer is based on (i) the known tendency for cancer cell glycoproteins to express simpler carbohydrate chains, such as STn, than do those from the corresponding normal cells [7, 23], (ii) the observation that monoclonal antibody B72.3 (which recognizes exclusively clustered STn epitopes) reacts strongly with a variety of human cancers but minimally or not at all with normal tissues, (iii) STn in cluster conformation is more cancer-specific in humans than are STn monomers, and (iv) the level of immunological tolerance against STn(c) may be lower than that against STn. Only clinical trials with an STn(c)-KLH vaccine can establish whether these ideas are correct.

We demonstrate here that mice immunized with STn or STn(c) conjugated to KLH with a two-carbon or MMCCH linker produced both IgM and IgG antibodies reacting strongly with OSM and with LS-C tumor cells. However STn(c)-KLH conjugated with MMCCH resulted in a high epitope ratio, the highest yield, and generally induced the highest titer of antibodies against OSM and LS-C. On the basis of these studies we have selected STn(c)-KLH conjugated with MMCCH plus immunological adjuvant QS-21 as the vaccine to be tested in clinical trials.

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