# Immunization of High-Risk Breast Cancer Patients with Clustered sTn-KLH Conjugate plus the Immunologic Adjuvant QS-21

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**Abstract**

**Purpose:** To determine the clinical toxicities and antibody response against sTn and tumor cells expressing sTn following immunization of high-risk breast cancer patients with clustered sTn-KLH [sTn(c)-KLH] conjugate plus QS-21.

**Experimental Design:** Twenty-seven patients with no evidence of disease and with a history of either stage IV no evidence of disease, rising tumor markers, stage II (≥4 positive axillary nodes), or stage III disease received a total of five injections each during weeks 1, 2, 3, 7, and 19. Immunizations consisted of sTn(c)-KLH conjugate containing 30, 10, 3, or 1 μg sTn(c) plus 100 μg QS-21. Induction of IgM and IgG antibodies against synthetic sTn(c) and natural sTn on ovine submaxillary mucin were measured before and after therapy. Fluorescence-activated cell sorting analyses assessed reactivity of antibodies to LSC and MCF-7 tumor cells.

**Results:** The most common toxicities were transient local skin reactions at the injection site and mild flu-like symptoms. All patients developed significant IgM and IgG antibody titers against sTn(c). Antibody titers against ovine submaxillary mucin were usually of lower titers. IgM reactivity with LSC tumor cells was observed in 21 patients and with MCF-7 cells in 13 patients. There was minimal IgG reactivity with LSC cells.

**Conclusion:** Immunization with sTn(c)-KLH conjugate plus QS-21 is well tolerated and immunogenic in high-risk breast cancer patients. Future trials will incorporate sTn(c) as a component of a multiple antigen vaccine.

Several structurally similar blood group–related carbohydrate antigens, including Thomsen-Freidenreich (TF), Tn, and sialyl Tn (sTn), attached to protein backbones of glycoproteins, are promising targets for vaccine therapy due to their widespread presence on the cell surface of human tumors (1, 2). In particular, the disaccharide sTn [NeuAcα(2→6)GalNAcα-0-Ser/Thr], O-linked to serine and threonine residues on mucins, is recognized by monoclonal antibodies (i.e., B72.3; refs. 3, 4) and expressed on tumors of breast, gastric, colon, pancreas, prostate, lung, endometrial, and ovarian origin (5–11). In this study, the evaluation of sTn as an antigenic target in breast cancer patients is explored.

There is limited expression of sTn on normal human cells (5, 8, 11, 12), although ovine submaxillary mucin (OSM) provides a natural source of sTn (13, 14). An association may exist between greater sTn expression on tumors and a poorer prognosis in breast cancer (15, 16); sTn may also be predictive of response to adjuvant chemotherapy in node-positive breast cancer (17).

Expression of sTn on normal cells is limited and primarily restricted to luminal surfaces (18). In tumors, this pattern is often disrupted. Abnormal glycosylation of tumor cell mucins results in shorter and fewer carbohydrate chains, allowing for greater exposure of antigens such as sTn; this may contribute to increased expression of sTn on tumors in comparison with normal cells (19–22). However, the role of sTn has not yet been clearly defined (23–27).

Development of a sTn-based vaccine requires consideration of several variables. First, the source of sTn can be natural (from OSM or human cells) or synthetic and influence the reactivity of various monoclonal antibodies (14). Second, sTn is not highly immunogenic because it is a carbohydrate as well as a "self-antigen". One approach to increase immunogenicity is conjugation of an antigen to keyhole limpet hemocyanin (KLH), an immunogenic protein carrier (20, 28), and the addition of immunologic adjuvants such as QS-21 (2, 29). Third, the conformation of sTn found on naturally occurring mucins may be different from that of synthetic sTn. For example, the monoclonal antibody B72.3, preferentially reactive with tumor cells over normal cells and OSM, has been shown to react primarily with clusters of sTn (5, 30, 31). A clustered formation of Tn antigens may have more relevant

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antigenicity than single Tn antigens (32), and this may be true for sTn as well.

Immunization of mice with either synthetic single sTn-KLH or clustered sTn-KLH [sTn(c)-KLH] conjugates plus QS-21 induced IgM and IgG antibodies reactive with OSM and the respective synthetic antigens as well as IgG antibodies reactive with sTn-positive tumor cells in both groups of mice (30). Inhibition assays revealed that the post-immunization sera were primarily inhibited by the particular construct in the vaccine. The similar reactivity of both sets of sera with OSM and sTn-positive tumor cells implies that the sera were reactive with either the clustered or the unclustered configuration. However, monoclonal antibodies, such as B72.3, that have the greatest specificity for tumor cells over normal cells react primarily with sTn clusters, suggesting that this is the most relevant target for vaccine construction.

The immunogenicity of sTn-KLH and sTn(c)-KLH vaccines in mice has been assessed using two different conjugation methods: by direct reductive amination or with a 4-(4-N-maleimidomethyl) cyclohexane-1-carboxyl hydrazide heterobifunctional linker (33). The 4-(4-N-maleimidomethyl) cyclohexane-1-carboxyl hydrazide linker was found to be the preferred method of conjugation for sTn(c) as it resulted in a more efficient yield and higher antibody titers against sTn(c). Other studies have shown inhibition of tumor growth in mice following immunization with constructs containing desialylated OSM (primarily Tn antigen; ref. 34) or synthetic TF (35).

The earliest clinical trials of sTn constructs evaluated patients with colorectal carcinoma following immunization with partially desialylated OSM plus either bacillus Calmette-Guerin or DETOX (36) and sTn-KLH plus either DETOX or QS-21 (37). The vaccines were well tolerated, and IgG and/or IgM antibodies reactive with the respective antigens were induced. Antibodies induced against sTn by the OSM constructs were of relatively low titers, whereas those induced by the sTn-KLH constructs were of greater titers. QS-21 seemed to be a more effective adjuvant than DETOX. Several studies of similar constructs in breast cancer patients have also shown evidence of immune response (20, 38, 39).

Based on (*a*) the reactivity of the monoclonal antibody B72.3 primarily with sTn(c), (*b*) the reactivity of B72.3 primarily with cancer cells and rarely normal cells, (*c*) clinical trials revealing immunogenicity of sTn-based immunizations in humans, and (*d*) the hope that higher titers of antibodies reactive with the tumor cell surface could be induced with sTn(c), we initiated a trial in high-risk breast cancer patients with sTn(c) conjugated to KLH plus QS-21. The objectives of this trial were to determine (*a*) clinical toxicities and (*b*) antibody response against sTn and tumor cells expressing sTn with various doses of sTn-KLH conjugate. Our preliminary data for nine patients showed an immune response with minimal toxicity (40), and we report here the final results for all 27 patients.

## Patients and Methods

***Patient population.*** Patients with a history of breast cancer who were without evidence of disease (NED) at the time of protocol entry were eligible. One of the following features was required (based on the *American Joint Committee on Cancer Staging System, 5th Edition*): stage IV NED, increasing CA15-3 or carcinoembryonic antigen levels (stages I-III), stage II (with ≥4 positive axillary lymph nodes), or stage III disease within 24 months of completing adjuvant therapy. A minimum of 4 weeks must have elapsed since surgery, chemotherapy, or radiation therapy and 6 weeks since immunotherapy. Exclusion criteria included pregnancy, seafood allergy, known autoimmune or immunodeficiency disorder, significant heart disease, other active cancers (excluding skin carcinomas), and Karnofsky performance status ≤ 80. The following were required: lymphocyte count ≥ $0.5 \times 10^6$/mL, WBC count ≥ 3,000 per μL, serum creatinine/aspartate aminotransferase/alkaline phosphatase ≤ 1.5× upper limit of normal, and γ-glutamyltranspeptidase ≤ 2× upper limit of normal. All patients signed an informed consent, approved by the Institutional Review Board and the Food and Drug Administration. Tumor sTn expression was not evaluated in this study.

***Immunization schedule and on-study evaluation.*** Each patient received a total of five s.c. injections (during weeks 1, 2, 3, 7, and 19), usually into the upper arm or thigh and rarely in the buttocks. Four groups of patients received sTn(c)-KLH conjugate containing either 30, 10, 3, or 1 μg sTn(c) plus 100 μg QS-21 in each dose. History and physical exams and carcinoembryonic antigen and CA15-3 levels were done during weeks 1, 7, and 19. Complete blood count and chemistry profiles were drawn before injection and then during weeks 3, 7, and 19. Blood for immune response was drawn during weeks 1, 2, 3, 5, 7, 9, 13, 19, and 21 and then every 3 months if feasible. Computerized tomography scans and bone scans were obtained before study and then during weeks 21 to 24.

***sTn(c)-KLH + QS-21 preparation.*** The synthetic sTn(c) was prepared at Biomira, Inc. and then transported to Memorial Sloan-Kettering Cancer Center. The cluster was prepared by the attachment of a synthetic sTn disaccharide to a serine. With Fmoc technology, three serine-sTn constructs were attached followed by conjugation of the last serine with a crotyl linker arm. The cluster was then conjugated to KLH by a 4-(4-N-maleimidomethyl) cyclohexane-1-carboxyl hydrazide linker for patient injections and to human serum albumin (HSA) for use in skin tests and *in vitro* analyses (41). The sTn cluster/KLH molar ratio was 409:1. Each vial contained either 30, 10, 3, or 1 μg sTn(c)-KLH plus 100 μg QS-21 (Antigenics, Inc.) in PBS. Samples underwent testing for toxicity and immunogenicity in mice as well as for sterility and endotoxin.

***Skin tests.*** General immune competence was evaluated by topical administration of dinitrochlorobenzene during weeks 1, 3, 9, and 21. The initial dose of dinitrochlorobenzene was 2,000 μg followed by lower doses based on the patient's reaction. The protocol was later amended to include administration of an i.d. skin test of 30 μg sTn(c) during weeks 1 and 9. The skin test responses were measured 48 h later by either a nurse or the patient.

***Serologic assays.*** Patient sera were evaluated for IgM and IgG antibodies against synthetic sTn(c), synthetic sTn, OSM, and tumor cells expressing sTn. A sTn-positive colon cancer cell line (LSC) and the breast cancer cell line MCF-7 were used for fluorescence-activated cell sorting analyses (19).

IgM and IgG antibodies were evaluated by ELISA assays according to the following procedure. NUNC 96-well ELISA plates were plated with 0.1 μg sTn(c)-HSA per well, in 60 μL carbonate buffer, and incubated overnight at 4°C. Following a PBS wash, unreactive sites were then blocked by incubation with 3% HSA for 2 h at 37°C. Serial dilutions of the patient's sera were then added to the wells, left at room temperature for 1 h, and then washed. Secondary antibodies, either alkaline phosphatase–labeled goat anti-human IgM or unlabeled mouse anti-human IgG, were added. For IgG detection, a tertiary antibody, alkaline phosphatase–labeled goat anti-mouse IgG (Southern Biotechnology), was then added. Following a 45-min incubation, the plates were washed, developed, and read at 405 nm on the ELISA reader. The highest serum dilution with an absorbance of ≥0.100 was recorded as the antibody titer.

Fluorescence-activated cell sorting analyses were done on pre- and post-immunization sera to determine reactivity of IgM or IgG antibodies with LSC cells and IgM antibodies with MCF-7 cells. A 1:20 dilution of patient sera was added to the tumor cells, washed, and then

**Table 1.** Patient characteristics

| | No. patients | | | |
|---|---|---|---|---|
| | Dose of sTn(c) (μg) | | | |
| | 30 | 10 | 3 | 1 |
| Total no. patients | 9 | 6 | 6 | 6 |
| Stage IV NED | 5 | 2 | 3 | 3 |
| Stage III | 1 | 4 | 1 | 2 |
| Stage II | 2 | 0 | 2 | 1 |
| Increased CA15-3 | 1 | 0 | 0 | 0 |
| Prior chemotherapy | | | | |
| Adjuvant only | 7 | 5 | 5 | 5 |
| Metastatic only | 0 | 0 | 0 | 1 |
| Adjuvant + metastatic | 2 | 1 | 1 | 0 |
| Prior radiotherapy | | | | |
| Adjuvant only | 4 | 3 | 3 | 3 |
| Metastatic only | 2 | 0 | 1 | 2 |
| Adjuvant + metastatic | 0 | 0 | 1 | 0 |
| Hormone therapy during the study | 6 | 4* | 2 | 4 |
| Age | | | | |
| Range (y) | 41-57 | 28-54 | 37-63 | 31-63 |
| Median (years) | 48 | 43.5 | 45 | 45 |

*One patient began tamoxifen while on study due to progression of disease.

mixed with 20 μL of a 1:25 dilution of goat anti-human IgM or IgG antibody (Southern Biotechnology) labeled with FITC. Following a 30-min incubation on ice, the cells were washed, and the percentage of positive cells was detected by a flow cytometer (FACScan, Becton and Dickinson). The positive controls were monoclonal antibodies B72.3 and CC49. Pre- and post-immunization sera were read together with the pre-immunization value calibrated to 10% positive cells.

Chromium release assays assessed complement-dependent cell cytotoxicity at various time points, before and after injection, based on availability of patient sera. Ten to 20 million MCF-7 or LSC tumor cells were washed in FCS-free media twice, resuspended in 500 μL of media, and incubated with 100 μCi Cr for 2 h at 37°C, during which the cells were shaken every 15 min. The cells were washed thrice in media to achieve a concentration of ~20,000 per well and plated in round-bottomed plates. The plates contained either 50 μL cells plus 50 μL monoclonal antibody as positive or 50 μL cells + serum (before and after) as experimental. The plates were incubated at 4°C on a shaker for 45 min. Human complement of a 1:5 dilution (resuspended in 1 mL of ice-cold water and diluted with 3% HSA) was added to each well at a volume of 100 μL. In the six control wells, Triton X-100 (10%, 20 μL) was added with media to a total volume of 200 μL. The plates were incubated for 2 h at 37°C and then centrifuged for 3 to 5 min. Then 30 μL of supernatant was removed for radioactivity counting.

***Criteria for cessation of treatment and dose reduction.*** Removal from the trial could occur with evidence of disease progression, grade 4 toxicity, or development of an autoimmune disease. Dose reductions were planned for grade ≥3 local or systemic toxicity.

***Biostatistical considerations.*** The trial was designed to accrue six patients per dose level (42). If no immune response was observed out of six patients, then the trial would be terminated (i.e., we would be >95% certain that the immunization would not induce response in <50% of patients). If one or more patients produced an immune response, then up to an additional six patients would be accrued to that dose level. If three or more patients induced an immune response, then the next cohort of six patients would be accrued at the next lowest dose level. Following immunization, a serologic response was defined as an antibody titer of ≥1:80 against OSM for those with no detectable baseline titer or a ≥8-fold increase for a baseline titer >0.

## Results

***Patients.*** Twenty-seven patients were entered on study between June 1996 and July 1997 (Table 1) The dominant sites of metastases included skin/chest wall ($n$ = 6), lymph nodes ($n$ = 3), ovaries ($n$ = 2), stomach ($n$ = 1), bone ($n$ = 1), and lung ($n$ = 1). One patient with a history of skin and nodal metastases was included twice. All metastases were confirmed by biopsy except for the bone lesion evident on radiographic imaging. The median time interval between the primary breast cancer diagnosis and the first injection was 116 weeks (range, 43-658 weeks). At the start of this trial, 15 patients were on hormone therapy, which they continued during the study, including tamoxifen ($n$ = 9), tamoxifen plus goserelin ($n$ = 1), anastrazole ($n$ = 4), and megestrol acetate ($n$ = 1). One patient developed disease progression after the fourth injection, in the uterus and omentum. She was clinically NED following surgery; tamoxifen was begun, and she continued on study.

All patients received five injections each according to schedule except one patient at the 30 μg dose level. She received the first three injections on schedule, but due to transportation issues, the fourth and fifth injections were given 1 day early. Serologic samples were drawn 1 day early for her week 5, 7, 13, 19, and 21 time points. However, for ease of interpretation of the data, her antibody and toxicity results are recorded as per the planned schedule.

***Toxicities.*** Toxicity data are available for 134 of 135 injections administered; one patient was lost to follow-up after the fifth injection (Table 2). A post-injection fever was graded as a drug fever under allergic reaction. There were no dose reductions in any patients as there were no grade 3 or 4 toxicities, excluding grade 3 lymphopenia, which was an acceptable pre-immunization value. The most common toxicities were transient local skin reactions at the injection site and mild flu-like symptoms. The local skin reactions, including pain, swelling, and erythema, usually resolved within 2 to 4 days and ranged from 1 to 10 days in duration. Two patients developed a small blister at the site of the third injection.

**Table 2.** Common toxicities

| Toxicity | Grade 1 | Grade 2 |
|---|---|---|
| Local skin reaction | 7 (6) | 116 (27) |
| Arthralgias | 12 (7) | 0 |
| Constipation | 2 (1) | 0 |
| Cough | 4 (3) | 0 |
| Diarrhea | 3 (3) | 0 |
| Dyspnea | 0 | 2 (2) |
| Fatigue | 40 (17) | 3 (3) |
| Fever | 14 (8) | 10 (6) |
| Headache | 28 (12) | 0 |
| Myalgias | 26 (14) | 2 (2) |
| Nausea | 9 (8) | 1 (1) |
| Vomiting | 5 (4) | 1 (1) |
| Rigors/chills | 12 (10) | 0 |
| Pruritis | 14 (11) | 0 |
| Urticaria | 1 (1) | 1 (1) |

NOTE: The number of episodes that occurred following all injections are outlined (134 of 135 injections are evaluable). The number of patients with the toxicity is noted in parentheses.

Following the second immunization, two patients noted a recall reaction at the first injection site. Flu-like symptoms usually resolved within a few days.

Six patients developed transient rashes while on study: one Herpes zoster, one dry skin, and three nonspecific skin changes (one rash near the injection site, one possible eczema, one chest wall and scalp rash of unclear etiology), possibly related to the immunizations. Another patient developed a photosensitivity reaction to the sun with desquamation of the skin and blisters, following the third immunization. A dermatology consultant concluded that this was due to phototoxicity from prior chemotherapy. One patient complained of transient abdominal pain after the fourth injection; stool was Hemoccult positive. Upper and lower endoscopies were negative, and subsequent stool Hemoccult several months later was negative. One patient developed joint aches ~4.5 years after the study. A rheumatoid factor was positive, and she is on anti-inflammatory agents for rheumatoid arthritis.

Hematologic changes occurred in the following number of patients: leukopenia, grade 1 ($n$ = 16) and grade 2 ($n$ = 3); anemia, grade 1 ($n$ = 7) and grade 2 ($n$ = 1); thrombocytopenia, grade 1 ($n$ = 12); and neutropenia, grade 1 ($n$ = 10) and grade 2 ($n$ = 4). Mild elevations of certain laboratory values were noted in the following number of patients and thought unrelated to the sTn(c)-KLH + QS-21: grade 1 alkaline phosphatase ($n$ = 5), grade 1 glucose ($n$ = 13), and grade 2 glucose ($n$ = 1). In addition, there were rare transient symptoms noted in the following number of patients thought unrelated to the sTn(c)-KLH + QS-21: anorexia ($n$ = 1), abnormal oral taste ($n$ = 1), near syncope ($n$ = 1), dysequilibrium ($n$ = 1), dizziness ($n$ = 1), dry mouth ($n$ = 1), blurry vision ($n$ = 1), paresthesias ($n$ = 2), and nasal congestion ($n$ = 2).

***Serologic response.*** All patients developed significant IgM and IgG antibody titers against sTn(c)-HSA (Fig. 1), and most patients developed significant antibody titers against OSM (Fig. 2). The 1 and 3 μg doses seemed to produce the highest sustained antibody titers, especially against sTn(c)-HSA. Sera were available in 24 patients to assess longer follow-up of antibody titers against sTn(c)-HSA. Sera obtained during weeks 42 to 97 (median, week 60) revealed median IgM titers of 1:240

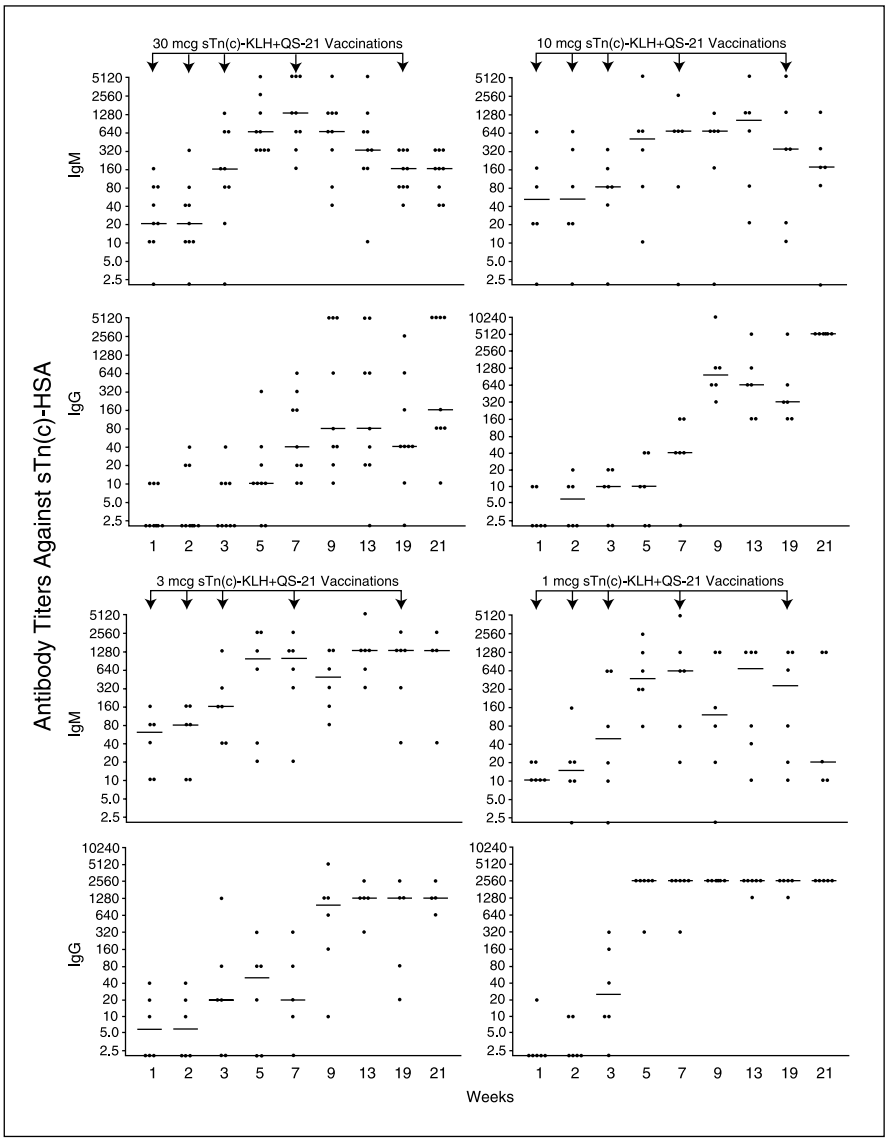


**Fig. 1.** IgM and IgG antibody titers against sTn(c)-HSA after immunization with sTn(c)-KLH conjugate + QS-21 for all dose levels. Bar, median.

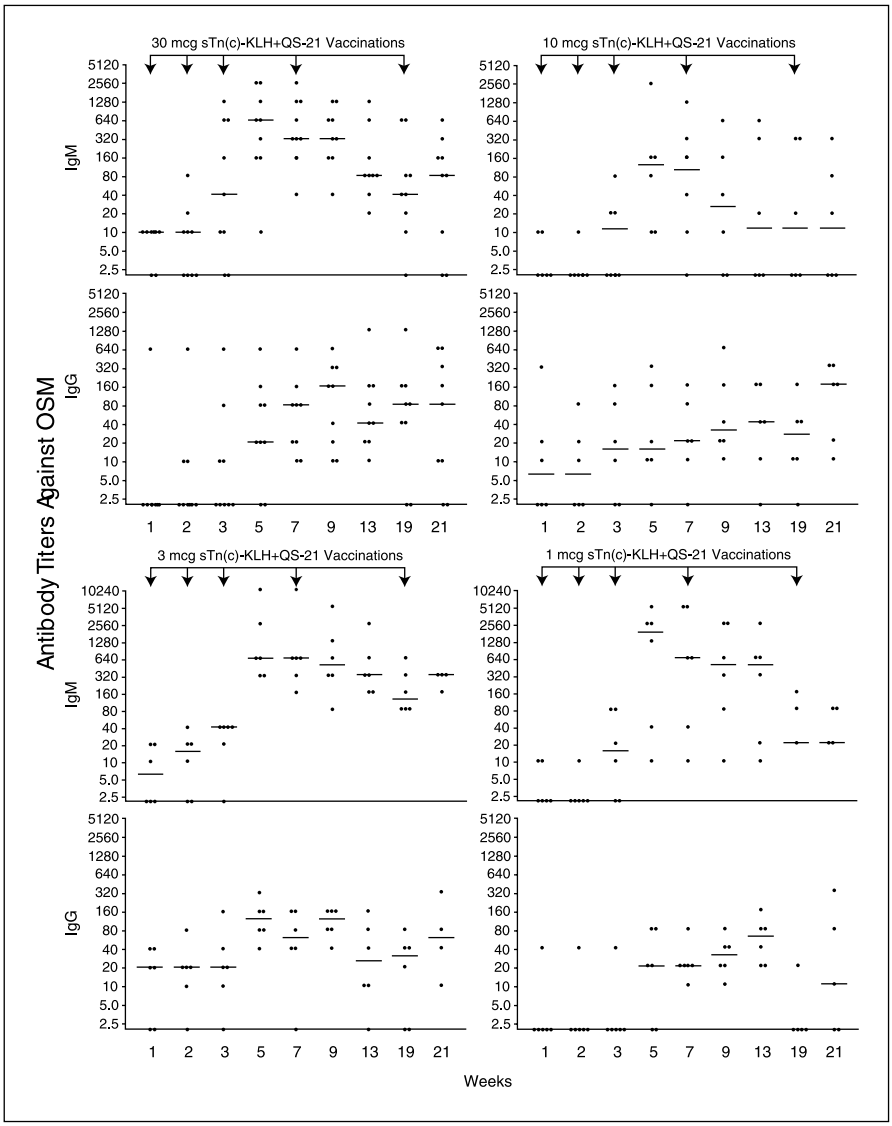


**Fig. 2.** IgM and IgG antibody titers against OSM after immunization with sTn(c)-KLH conjugate + QS-21 for all dose levels. Bar, median.

(range, 1:10 to 1:5120) and median IgG titers of 1:1,280 (range, 0 to 1:5,120). These are similar to the week 21 values.

Antibodies against unclustered sTn-HSA were subsequently evaluated in most patients at fewer time points. Significant IgM and IgG antibody titers against sTn-HSA (titer of ≥1:80 or ≥8-fold above baseline) were noted in 17 of 27 patients and in 9 of 23 patients, respectively (data not shown).

Reactivity of IgM and IgG antibodies with LSC and MCF-7 tumor cells was considered significant if there was a 3-fold increase in the percentage of tumor cells reactive with the antibody. IgM reactivity against LSC cells was significantly elevated in 21 patients, with no difference between the four doses (Table 3). IgM reactivity against MCF-7 tumor cells was observed less frequently (Table 4). IgG reactivity with LSC tumor cells was noted in 3 of 27 patients (data not shown). Despite this significant cell surface reactivity, there was no definite evidence of consistent complement-dependent cytotoxicity against LSC cells in 22 patients or against MCF-7 cells in 27 patients (data not shown).

***Skin tests.*** All patients had skin reactivity to dinitrochlorobenzene. The week 1 sTn skin test was completely negative in 24 of 26 patients. One patient did not report the result; a 0.5-cm pink area was noted in another patient. At week 9, the sTn skin test was completely negative in 25 of 27 patients. One patient reported a 5-cm skin reaction; a 1-cm pink area was noted in another patient.

***Clinical course.*** During the study, patients were monitored for evidence of disease by radiographic studies and physical examinations, although clinical response was not an end point. The median interval from the date of the first immunization to the date of last follow-up or death for all patients is 339 weeks (range, 46-383 weeks). At the conclusion of the trial, of 25 evaluable patients, 21 remained NED, and 4 had disease progression in the omentum, lung, mesentery, and skin, respectively. Of those with progression, three had stage IV disease, and one had increased tumor markers at the start of the trial.

Long-term follow-up is available for 26 patients. Of these, 8 patients have died, 6 patients are alive following a subsequent metastatic recurrence, and 12 patients remain NED. One developed a contralateral primary breast cancer, and one developed a new intraductal cancer, but both are considered NED in terms of their original disease. One patient who refused

**Table 3.** IgM antibodies: fluorescence-activated cell sorting analysis against LSC tumor cells with sera obtained before and after immunizations compared with ELISA reactivity against sTn(c)-HSA

| 30 μg | | | | | 10 μg | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Patient no. | Week no. | ELISA titers | % Cells positive | MFI | Patient no. | Week no. | ELISA titers | % Cells positive | MFI |
| 1 | 1 | 40 | **10.48** | 27.79 | 10 | 1 | 20 | **9.91** | 28.52 |
| | 5 | 320 | **64.8** | 76.25 | | 9 | 640 | **59.87** | 85.38 |
| 2 | 1 | 80 | **9.96** | 52.13 | 11 | 1 | 640 | 10.6 | 119.1 |
| | 9 | 1280 | **39.07** | 92.79 | | 21 | 80 | 13.22 | 189.26 |
| 3 | 1 | 20 | **10.46** | 26.12 | 12 | 1 | 20 | **9.94** | 26.69 |
| | 21 | 320 | **93.15** | 94.44 | | 5 | 5120 | **99.63** | 366.93 |
| 4 | 1 | 10 | **10.72** | 33.16 | 13 | 1 | 80 | 10.39 | 40.8 |
| | 5 | 1280 | **93.38** | 117.15 | | 9 | 160 | 6.34 | 19.32 |
| 5 | 1 | 80 | 10.81 | 42.71 | 14 | 1 | 160 | **10.21** | 68.01 |
| | 7 | 1280 | 22.06 | 128.12 | | 5 | 640 | **32.23** | 108.47 |
| 6 | 1 | 0 | **10.49** | 10.73 | 15 | 1 | 0 | 9.96 | 52.78 |
| | 9 | 320 | **90.57** | 48.81 | | 9 | 0 | 12.27 | 51.43 |
| 7 | 1 | 20 | **9.94** | 10.78 | | | | | |
| | 5 | 2560 | **98.26** | 176.28 | | | | | |
| 8 | 1 | 160 | **9.41** | 22.79 | | | | | |
| | 5 | 5120 | **95.96** | 131.60 | | | | | |
| 9 | 1 | 10 | **10.17** | 20.22 | | | | | |
| | 5 | 640 | **71.31** | 52.41 | | | | | |

NOTE: The numbers in bold indicate a positive response.
Abbreviation: MFI, mean fluorescence intensity.

scans at the study conclusion remains NED and is included among the 26 patients. Of the 21 patients who were NED at the conclusion of the trial, 10 patients developed a subsequent recurrence at a median interval of 91.5 weeks (range, 52-257 weeks) from the date of the first immunization, and 5 have died of disease. Of the 13 patients with stage IV disease at the start of the trial, 7 remain alive.

## Discussion

This trial is one of a series at our center designed to evaluate the toxicity and immune response to immunization with carbohydrate or peptide constructs in high-risk breast cancer patients (43, 44). The toxicities are similar to those observed in our previous trials, with common transient local

**Table 4.** IgM Antibodies: fluorescence-activated cell sorting analysis against MCF-7 tumor cells with sera obtained before and after immunizations compared with ELISA reactivity against sTn(c)-HSA

| 30 μg | | | | | 10 μg | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Patient no. | Week no. | ELISA titers | % Cells positive | MFI | Patient no. | Week no. | ELISA titers | % Cells positive | MFI |
| 1 | 1 | 40 | 10.93 | 27.76 | 10 | 2 | 20 | **10.27** | 24.8 |
| | 5 | 320 | 14.18 | 27.26 | | 9 | 640 | **40.21** | 53.6 |
| 2 | 2 | 80 | 9.96 | 23.00 | 11 | 1 | 640 | 10.1 | 22.01 |
| | 9 | 1280 | 12.96 | 24.63 | | 21 | 80 | 5.98 | 13.58 |
| 3 | 2 | 20 | 10.13 | 19.25 | 12 | 2 | 20 | **10.4** | 14.58 |
| | 21 | 320 | 13.52 | 16.92 | | 7 | 2560 | **56.82** | 30.66 |
| 4 | 2 | 10 | 10.76 | 11.69 | 13 | 2 | 80 | 10.03 | 45.02 |
| | 5 | 1280 | 22.06 | 13.81 | | 9 | 160 | 7.05 | 34.5 |
| 5 | 2 | 40 | 10.04 | 14.59 | 14 | 2 | 320 | 9.28 | 15.09 |
| | 7 | 1280 | 15.43 | 17.60 | | 5 | 640 | 8.38 | 11.89 |
| 6 | 2 | 0 | **9.74** | 19.38 | 15 | 1 | 0 | 10.2 | 37.72 |
| | 13 | 160 | **67.55** | 79.85 | | 9 | 0 | 15.06 | 43.43 |
| 7 | 2 | 10 | **10** | 31.26 | | | | | |
| | 5 | 2560 | **35.37** | 51.95 | | | | | |
| 8 | 2 | 320 | **9.67** | 25.03 | | | | | |
| | 7 | 5120 | **31.12** | 41.64 | | | | | |
| 9 | 2 | 10 | 10.23 | 16.06 | | | | | |
| | 5 | 640 | 15.16 | 15.60 | | | | | |

NOTE: The numbers in bold indicate a positive response.

**Table 3.** IgM antibodies: fluorescence-activated cell sorting analysis against LSC tumor cells with sera obtained before and after immunizations compared with ELISA reactivity against sTn(c)-HSA (Cont'd)

| 3 μg | | | | | 1 μg | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Patient no. | Week no. | ELISA titers | % Cells positive | MFI | Patient no. | Week no. | ELISA titers | % Cells positive | MFI |
| 16 | 1 | 40 | **9.96** | 49.67 | 22 | 1 | 20 | **9.74** | 26.51 |
| | 5 | 2560 | **80.78** | 262.78 | | 5 | 1280 | **88.86** | 99.07 |
| 17 | 1 | 80 | **10.79** | 100.71 | 23 | 1 | 20 | 9.98 | 47.4 |
| | 5 | 640 | **89.85** | 386.16 | | 9 | 80 | 7.24 | 25.01 |
| 18 | 1 | 10 | 10.61 | 195.07 | 24 | 1 | 10 | **9.6** | 28.54 |
| | 5 | 20 | 9.68 | 212.4 | | 9 | 20 | **30.11** | 47.87 |
| 19 | 1 | 10 | **10.88** | 58.96 | 25 | 1 | 10 | **10.5** | 20.38 |
| | 5 | 2560 | **89.07** | 244.97 | | 5 | 640 | **96.33** | 446.98 |
| 20 | 1 | 160 | **9.71** | 58.53 | 26 | 1 | 10 | **10.8** | 84.81 |
| | 5 | 40 | **70.49** | 128.41 | | 5 | 320 | **90.95** | 246.14 |
| 21 | 1 | 80 | **9.75** | 74.85 | 27 | 1 | 10 | **9.67** | 48.1 |
| | 9 | 1280 | **98.87** | 47.3 | | 7 | 5120 | **88.11** | 194.24 |

reactions at the injection site. No ulceration or granuloma formation was observed, in contrast to studies with bacillus Calmette-Guerin or DETOX. The systemic flu-like symptoms, likely due to the QS-21, usually resolved within a few days. Although sTn is present on a limited number of normal cells and in a distribution thought less accessible to the immune system, an autoimmune reaction is always a theoretical concern. During the trial, there was no definite evidence of autoimmune-mediated reactions. The development of rheumatoid arthritis nearly 5 years later in one patient is unlikely directly related to the injections. However, immunizations could theoretically exacerbate an unrecognized autoimmune illness. Therefore, long-term follow-up of patients is warranted.

IgM and IgG antibodies strongly reactive with sTn(c)-HSA and OSM were observed in most patients following immunization with sTn(c)-KLH plus QS-21. Both IgM and IgG antibody titers against sTn(c)-HSA were generally elevated by

**Table 4.** IgM Antibodies: fluorescence-activated cell sorting analysis against MCF-7 tumor cells with sera obtained before and after immunizations compared with ELISA reactivity against sTn(c)-HSA (Cont'd)

| 3 μg | | | | | 1 μg | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Patient no. | Week no. | ELISA titers | % Cells positive | MFI | Patient no. | Week no. | ELISA titers | % Cells positive | MFI |
| 16 | 2 | 80 | 10.52 | 24.95 | 22 | 1 | 20 | 10.06 | 41.13 |
| | 5 | 2560 | 12.06 | 25.24 | | 5 | 1280 | 30.41 | 55.07 |
| 17 | 1 | 80 | **9.69** | 47.01 | 23 | 1 | 20 | 9.8 | 48.25 |
| | 5 | 640 | **44.56** | 93.21 | | 9 | 80 | 4.98 | 29.28 |
| 18 | 1 | 10 | 10.41 | 83.93 | 24 | 1 | 10 | 10.42 | 47.67 |
| | 5 | 20 | 9.81 | 81.83 | | 9 | 20 | 6.22 | 47.66 |
| 19 | 1 | 10 | **10.92** | 51.07 | 25 | 1 | 10 | **11.31** | 38.47 |
| | 5 | 2560 | **47.79** | 97.81 | | 5 | 640 | **92.55** | 198.86 |
| 20 | 2 | 160 | **10.8** | 56.37 | 26 | 1 | 10 | **9.56** | 41.79 |
| | 21 | 1280 | **48.51** | 127.31 | | 5 | 320 | **31.18** | 117.08 |
| 21 | 1 | 80 | **9.56** | 49.13 | 27 | 1 | 10 | **10.71** | 54.24 |
| | 9 | 1280 | **39.12** | 85.56 | | 7 | 5120 | **55.55** | 118.29 |

week 7 and, in the majority of patients, remained elevated for at least 12 months from the first immunization. This is similar to the antibody response observed after vaccination with MUC-1-KLH conjugates that remained positive for at least 12 months but different than the response noted after vaccination with ganglioside or neutral glycolipid-KLH conjugates that rarely lasted for more than 6 months without repeated booster immunizations (43, 45, 46). It is possible that future immunization schedules will include intermittent "boosters" based on the patient's clinical status.

The IgM and especially IgG antibody titers against sTn(c)-HSA were most consistently elevated at the 3 and 1 μg doses. The significance of this is unclear because there did not seem to be a dose effect with antibodies against OSM (the more natural form of sTn) or with reactivity against tumor cells expressing sTn. It is possible that some of the high titer IgG response against sTn-HSA includes artifactual epitopes, such as the crotyl linker arm, used to link sTn(c) to both KLH and HSA. Antibody titers against OSM were generally lower than the antibody titers against sTn(c). This finding is consistent with preclinical data showing that induced antibodies tend to be most reactive with the specific antigen used in the immunization (30).

There was significant binding of IgM antibodies with LSC tumor cells but minimal reactivity of IgG antibodies. This finding was observed in our prior trials and may be due to affinity of the antibodies. If affinity maturation failed to occur because sTn is a carbohydrate autoantigen, then higher affinity of IgM antibodies would result because of their pentameric structure. There was also less binding of IgM antibodies to MCF-7 cells in comparison with LSC cells. This might be explained by greater sTn expression on LSC cells compared with MCF-7 cells as well as variation of the epitopes on these cells. In addition, MCF-7 reactivity was done after the LSC testing, and during this time, some antibody degradation may have occurred. Importantly, reactivity of antibodies induced by sTn (c)-KLH plus QS-21 against both OSM and sTn-positive tumor cells was more consistent and more potent than antibodies induced by our previous unclustered sTn-KLH or OSM vaccines (36, 37). This is consistent with preclinical data showing that reactivity of B72.3 and CC49 (clinically relevant antibodies against sTn-positive tumor cells) was stronger against sTn(c)-HSA than sTn-HSA (30). This was the basis for evaluating the clustered form of sTn in this trial.

Complement binding and activation is a correlate of cell surface–binding IgM antibodies such as those induced in this study. The lack of complement-dependent cytotoxicity on target cells despite strong cell surface binding by fluorescence-activated cell sorting was not unexpected. We have previously shown that monoclonal antibodies and immune sera against antigens on mucins (i.e., sTn, Tn, and TF) were able to activate complement at the cell surface but that this did not result in complement-mediated lysis (47). The location of complement activation by antibodies against sTn and other mucin antigens may be too far from the cell surface for optimal activation of complement-induced cytotoxicity. However, preclinical mouse models show inhibition of tumor growth following induction of antibodies against mucin-related antigens (34, 35). Therefore, an antitumor response for mucin antigens may not always require complement-mediated lysis but may involve mechanisms such as opsonization and antibody-dependent cellular cytotoxicity.

Immunization with unclustered sTn constructs in other breast cancer trials has shown evidence of an immune and clinical response (20, 38). Cyclophosphamide was administered before the first injection in some trials, based on its apparent decrease of suppressor cell activity and the potential immunosuppressive effect of mucins (25, 48). Similar to our study, these trials show the induction of antibodies most reactive with the immunizing antigen sTn but also with some reactivity against OSM. It is unclear whether the cyclophosphamide contributed to the clinical response.

Our trial was not designed to assess clinical benefit or to correlate immune response with clinical outcome due to the small numbers of patients, concomitant hormone therapy, and administration of various prior therapeutic regimens. At a median follow-up of 339 weeks, 18 of 26 evaluable patients were alive. At this time point, of the 5 patients with stage II disease, 4 patients remained NED, and 1 was lost to follow-up. Of the 8 patients with stage III disease, 5 remained NED, 1 died, and 2 were alive with recurrent disease. Of the 13 patients with stage IV disease, 7 were alive. These data are not unexpected and show a group of patients with stage IV disease who may have a prolonged clinical course. It is not possible from this study to determine whether the vaccine had any significant effect on recurrence or survival. Evaluation of clinical benefit will require larger number of patients as the target population often has no definite evidence of disease. Patients with no disease or minimal disease are more likely to complete a series of immunizations than patients with rapidly progressive metastatic disease.

However, improved survival has been suggested in patients who develop antibodies against sTn or OSM following immunization with the Theratope vaccine (sTn-KLH plus DETOX; ref. 49). A recent trial in metastatic breast cancer compared the Theratope construct versus DETOX plus KLH (50). For the 1,028 randomized patients, preliminarily, there was no significant difference between time to disease progression or overall survival, although there was a trend toward improved outcome for those receiving hormone therapy and the Theratope vaccine. Final analysis of this study is still pending. However, additional studies with this agent are focusing on metastatic breast cancer patients who are receiving hormone therapy.

Numerous research topics remain regarding immunizations for breast cancer, including the optimal antigen/s, dose of antigen, immune adjuvant, schedule of administration, time interval since prior chemotherapy (39), and trial design. However, we can conclude that sTn(c)-KLH at sTn(c) doses between 1 and 30 μg plus QS-21 is well tolerated and immunogenic in high-risk breast cancer patients. Antibodies against sTn(c) and OSM and antibody reactivity with sTn-positive tumor cells were observed. Based on these results, we are evaluating the 3 μg dose of sTn(c)-KLH as a component of a polyvalent construct. Future studies will require larger numbers of patients to determine clinical benefit.

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