# A Fully Synthetic Globo H Carbohydrate Vaccine Induces a Focused Humoral Response in Prostate Cancer Patients: A Proof of Principle**

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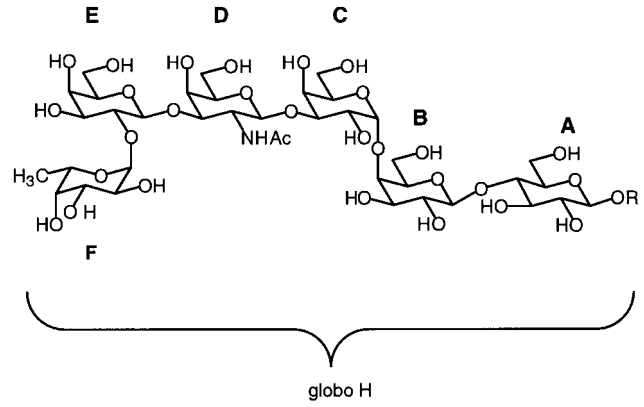
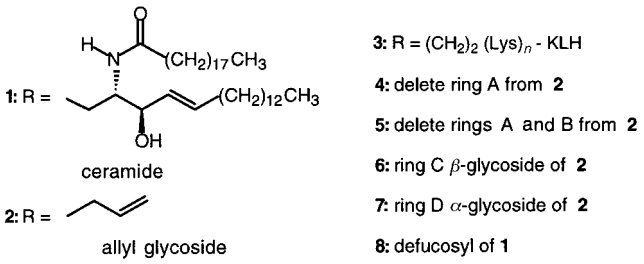
The enlistment of the formidable resources of the human immune system against cancerous lesions has been a long-standing vision in medicine.[1, 2] Ideally, a vaccine containing a particular tumor-associated antigen or a range of cell-surface antigens, presented in an effective immunostimulatory context, would trigger active immunity against cancer cells expressing counterpart structures on their surfaces.[3] The focus of our investigation is the development of a vaccine strategy that would be active in an adjuvant or minimum disease setting, providing enhanced protection against micrometastasis in a context where the primary tumor has been eliminated through surgery, radiation, or chemotherapy. The primary targets of our studies have been carbohydrate-based antigens such as glycolipids, or glycoproteins (including mucins), expressed on the accessible surfaces of tumor cells.[4, 5]

In this particular study, we have concentrated on the globo H tumor antigen. This antigen was first identified chemically from breast tumor extracts by Hakomori et al.[6] It was immunocharacterized by Colnaghi et al. (mAb MBr1),[7] and more recently by Lloyd et al. (mAb VK-9)[8] (mAb = monoclonal antibody). By the criteria of immunohistology, globo H was identified on a number of human cancers (including prostate and breast cancer) and in a restricted number of normal epithelial tissues.[9]

To proceed in a productive fashion, it was first necessary for us to accomplish a total synthesis of the globo H antigen (**1**) and properly conjugated immunogenic variants thereof, in adequate quantities for preclinical studies. These goals were




achieved by drawing heavily on the principles of glycal assembly (Scheme 1).[10, 11] The allyl group in the fully synthetic glycoside (**2**) was used as a spacer element, and as a point of chemical access for conjugating the hexasaccharide antigen of globo H to the carrier protein keyhole limpet hemocyanin (KLH; see structure **3**).

Following favorable serological and cell-surface reactivity in the vaccination of mice with construct **3**,[12] and after successful scale-ups in the total synthesis, a clinical trial using fully synthetic globo H vaccines in prostate cancer patients was launched. The vaccine construct **3**, in conjunction with the QS-21 adjuvant, had proven to be particularly immunogenic in the murine setting for eliciting globo H-specific responses.[12]

It should be emphasized that in progressing from a murine to a human setting for the vaccination, two potential risks had to be faced. Human sera and cell-surface glycoproteins present related structures (such as Lewis blood group determinants and, indeed, low levels of globosides). Hence, in the human clinical setting, there were potential issues of immunotolerance or possibly autoimmune responses to be addressed which were not pertinent to the earlier experiments with mice.

Here we report on encouraging early results from our clinical investigation using conjugate system **3** in tandem with QS-21. Five patients with progressive and recurrent prostate cancer received the conjugate vaccine, containing 30 μg of

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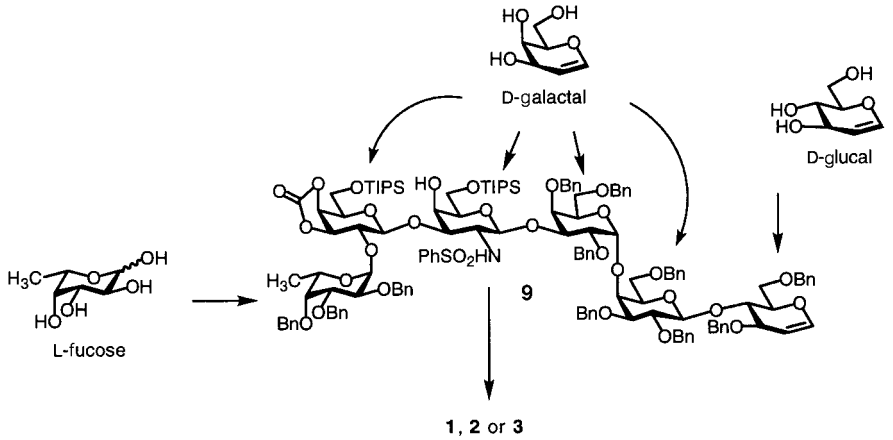


Scheme 1. The total synthesis of globo H hexasaccharide showing the logic of glycal assembly. Bn = benzyl, TIPS = triisopropylsilyl.

globo H plus QS-21, according to defined clinical protocols. Their sera were submitted for detailed analyses and evaluation. By ELISA (enzyme-linked immunosorbent assay), no detectable IgM or IgG antibodies against synthetic globo H were present prior to vaccination (Figure 1). The pre- and

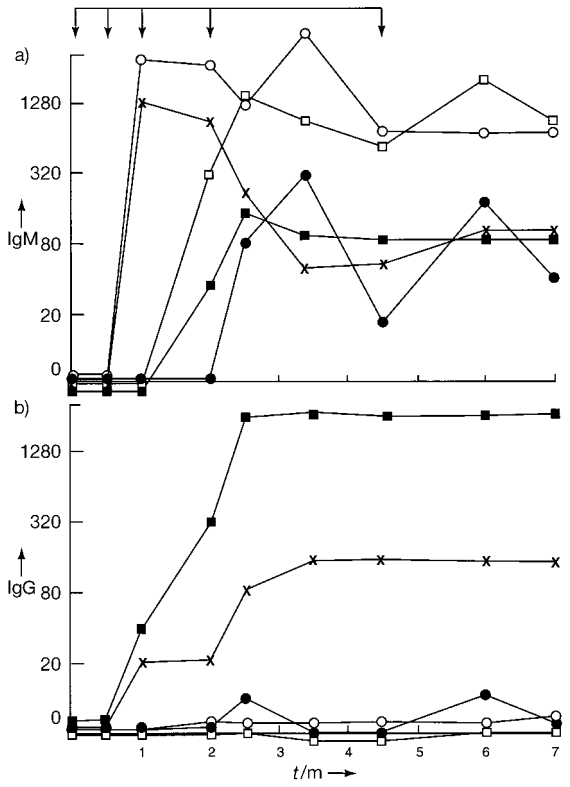


Figure 1. Time course of the induction of antibodies in five patients immunized with globo H – KLH conjugate and QS-21: a) IgM titer, b) IgG titer. ●: patient 1; × : patient 2; ○: patient 3; □: patient 4; ■: patient 5. The y axis shows the reciprocal titer against globo H by ELISA, and the x axis the time *t* in months. The arrows pointing down indicate vaccinations with globo H – KLH (30 μg) plus QS-21.

post-vaccination IgM and IgG ELISA titers against globo H – ceramide in sera from all five patients are shown in Figure 1. Subsequent to vaccination, all five patients produced a strong IgM response, while two concurrently generated a high IgG response. The specificity of these antibodies for globo H (derived from synthesis) or for globo H in prostate cancer cell extract from tumor or biopsy, as well as breast cancer biopsy specimens, was analyzed by immune thin layer chromatography (ITLC, Figure 2; results of all five patients are summarized in Table 1). Though the vaccines were based on a synthetic globo H – protein conjugate, the postvaccination sera recognized both synthetic and tumor-derived globo H – ceramide. A similar finding has been described for the VK-9 anti-

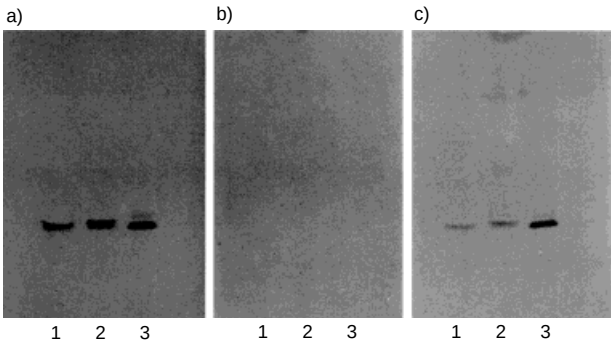


Figure 2. Immune thin-layer chromatography (ITLC) with synthetic and natural antigens and sera from patient 2 vaccinated with globo H – KLH conjugate. In each case, antigen from prostate cancer extract, antigen from breast cancer extract, and synthetic globo-H antigen, is applied in lanes 1, 2, and 3, respectively. Detection was achieved a) with a solution of the antibody mAb MBr1, b) with prevaccination sera, and c) with post vaccination sera.

body.[8, 10] By contrast, the sera failed to react with melanoma biopsy specimen extracts, which contain various glycolipids but are globo H negative.

Two different types of ELISA inhibition assays were carried out to determine the specificity of the anti-globo H antibodies in the immunized patients: 1) inhibition of reactivity against globo H – ceramide with structurally related antigens previously obtained by total synthesis in our laboratory[10, 12] as well as structurally unrelated antigens as controls; and 2) inhibition (absorption) by globo H-positive and -negative cell lines. The results of these studies (shown in Figure 3 a, b ) demonstrate that globo H – ceramide inhibits anti-globo H reactivity more efficiently than any of the other structurally related congeners obtained through synthesis. For instance, globo H allyl glycoside **2** at a concentration of 500 μM inhibited 40 % of the anti-globo H antibody activity while globo H-ceramide **1** at the same concentration inhibited 90 % of the reactivity. This finding is particularly interesting in that the immunizing antigen (globo H – KLH) lacked the ceramide moiety. All truncated oligosaccharide isomers of globo H previously prepared by synthesis[13] were also recognized, though less so than **1**. For example, SSEA – 3 (**8**; SSEA = stage-specific embryonic antigen), which lacks the fucose residue, also

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Table 1. Summary of immune thin layer chromatography with synthetic and natural antigens and sera from five patients vaccinated with globo H–KLH conjugate.[a]

| Patient | globo H–ceramide | | Prostate CA extract | | Breast CA extract | | Melanoma extract | |
|---|---|---|---|---|---|---|---|---|
| | pre | post | pre | post | pre | post | pre | post |
| 1 | − | +++ | − | + | − | + | − | − |
| 2 | − | +++ | − | ++ | − | ++ | − | − |
| 3 | − | ++ | − | ++ | − | ++ | − | − |
| 4 | − | ++ | − | ++ | − | ++ | − | − |
| 5 | − | +++ | − | ++ | − | ++ | − | − |
| mAb Mbr1[b] | ++++ | | +++ | | +++ | | − | |

[a] CA = carcinoma; pre = before vaccination, post = after vaccination. [b] Reference antibody (positive control).

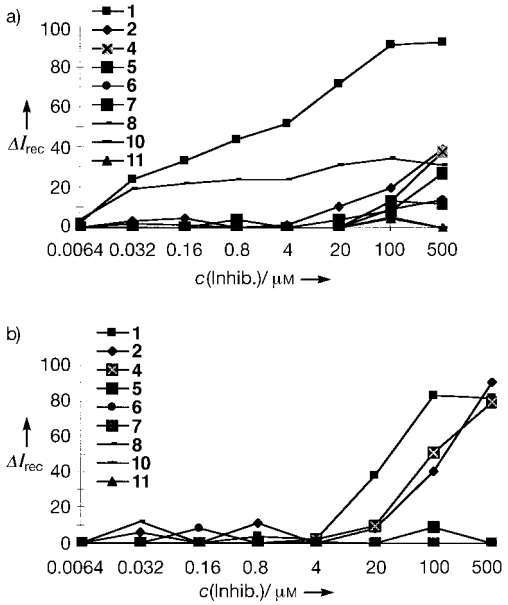


Figure 3. Analysis of the specificity of anti-globo H antiserum by inhibition assays. ELISA reactivity of serum with globo H-ceramide inhibited with compounds **1**, **2**, **4**–**8**, **10**, **11**: a) IgM antibody response, b) IgG antibody response. $\Delta I_{rec}$ = inhibition (%), *c* (Inhib.) = concentration of the inhibitor.

inhibited 30% of the antibody reactivity (Figure 3a). As a control, the unrelated glycolipid GD3 **10** containing the ceramide chain, but otherwise lacking any resemblance to the carbohydrate sector of the globo H and synthetic Le$^y$–allyl glycoside **11**, showed no inhibition.

GD3 = NeuAc-$\alpha$(2→8)-NeuAc-(2→3)-Gal-$\beta$(1→4)Glc-$\beta$(1–1′)-Cer **10**

Le$^y$ = Fuc-$\alpha$(1→2)-Gal-$\beta$(1→4)-(Fuc-$\alpha$(1→3))-GlcNAc-$\beta$(1→3)-Gal-$\beta$-*O*-Allyl **11**

The IgG response, however, was found to be quite different. In the two antisera demonstrating IgG ELISA reactivity, synthetic hexasaccharides **1** and **2**, as well as a pentasaccharide analogue (see compound **4**) derived from the nonreducing end of the molecule effectively inhibited binding in this assay (Figure 3b). No inhibition was seen with SSEA–3 **8**, which lacks the fucose residue or the tetrasaccharide **5**, which lacks the lactose moiety. Thus, IgG antibodies from both antisera appear to mainly recognize an epitope area encompassing five nonreducing terminal carbohydrate units (see segments B–F).

The lack of recognition of Lewis$^Y$ antigen **11** is particularly noteworthy since many of the constituent building blocks in **1** are also present in **11**. Clearly, the specificity for **1** arises from the difference in the structural and stereochemical connectivity of the antigenic subunits. The general message which comes through from the inhibitory characteristics of our fully synthetic various terminal probe structures is that of a polyclonal, but focused, response against various portions of globo H. The results show that a fucosylated tetra- or pentasaccharide structure is required for an optimal anti-globo H response.

Encouraged by these results, we evaluated whether antibodies elicited by synthetic vaccine **3** recognize the globo H antigen in its natural context, that is, the cell surface. This type of challenge is a crucial milestone in the progression of antitumor vaccines. Two assays were developed to measure the cell surface binding. In the first assay, sera were mixed with globo H-positive (MCF-7) and globo H-negative cell lines (SK-MEL-28). More than 50% of the ELISA reactivity against globo H–ceramide was lost following incubation with MCF-7 cells in all patients. No decrease in binding activity was observed following incubation with globo H-negative SK-MEL-28 melanoma cells. Comparable results were obtained in control experiments with mAb VK-9.

Furthermore, the cell-surface reactivity of anti-globo H antibodies was tested by flow cytometry (Figure 4). As judged by this assay, sera before vaccination showed very low

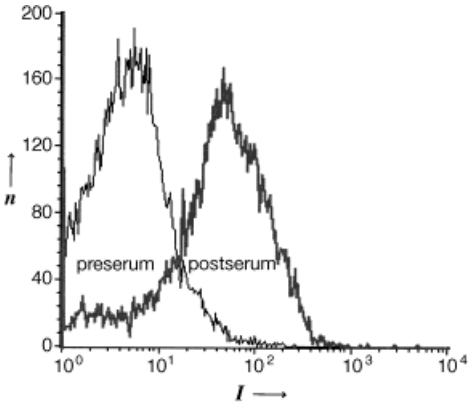


Figure 4. Reactivity of representative pre- and postvaccination sera with MCF-7 cells by flow cytometry. *n* = relative MCF-7 cell number (counts). *I* = fluorescence intensity.

reactivities with cell surfaces. However, sera drawn after the fourth vaccination showed an increase of IgM reactivity with MCF-7 breast cancer cells ranging from 11 to 97%. In a

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similar vein, the increase of the IgG antibody cell surface reactivites ranged from 15 to 35%.

As the last element of the serologic evaluation following vaccination, we also tested the anti-globo H sera for their ability to mediate complement-dependent cytotoxicity (CDC). Three of the five postvaccination sera showed strong CDC to MCF-7 cells. In control experiments, we found that 1) the same sera in the absence of complement, 2) complement without sera, and 3) prevaccination sera with complement failed to exhibit CDC under the same conditions.

In summary, our globo H glycoconjugate based vaccine (**3**) induces strong and well targeted humoral immune responses in patients. The resultant antibodies not only recognize the synthetic antigens (**1** and **2**) but also globo H-positive tumor biopsy extracts and tumor tissues. The antisera following vaccination successfully mediated complement-induced lysis of relevant cancer cells.

We note that, in a recent study in melanoma, antibodies induced in the setting of microscopic disease seem to be associated with decreased tumor outgrowth and appear to favor longer patient survival times.[14] In this context, the serological findings described here through vaccination of prostate cancer patients with the globo H vaccine portend a clinical advantage.

A formal full clinical report on this trial and a related globo H-directed breast cancer trial, conducted in larger patient populations, will be presented separately. Following the important "proof of principle" findings described herein, and other clinical indications associated with tumor-related complex carbohydrate antigens to be described soon, new trials in prostate and breast cancer patients using totally synthetic carbohydrate vaccines are in various stages of development.

### *Experimental Section*

Vaccine preparation and clinical protocol:

The globo H–KLH conjugate was prepared in a manner similar to sialyl Tn cluster–KLH conjugate, as previously described.[15] Patients with progressive prostate cancer that had a minimum of three rising prostate-specific antigen (PSA) values were vaccinated with globo H-KLH vaccine containing 30 μg of globo H and 100 μg of QS-21. Three vaccinations were administered subcutaneously at one week intervals. Two additional vaccinations were administered at week 7 and 19. Peripheral blood (20–30 mL) was drawn immediately before each vaccination, and two weeks after the fourth and fifth vaccinations. The sera obtained from prevaccination and two weeks after the third, fourth, and fifth vaccinations of all patients were tested for antibodies against globo H–ceramide, truncated globo H analogues, tumor extracts, and the globo H-positive MCF-7 cell-line.

Serological analysis:

ELISA: ELISAs were performed as described previously.[8, 16] ELISA plates were coated with globo H–ceramide at 0.1 μg per well. Serially diluted patient serum was added to wells of the coated plates, and antibody titer was defined as the highest serum dilution showing an absorbance 0.1 or greater over that of normal patient sera.

Immune thin layer chromatography (ITLC): Immune staining of synthetic globo H–ceramide, and the neutral glycolipid extract obtained from breast and prostate cancers with patient sera or mAb MBr1, was performed after separation on HPTLC silica gel glass plates as previously described.[8, 16] Patient sera diluted appropriately in phosphate-buffered saline (PBS), and anti-human IgG or IgM antibodies conjugated with horseradish peroxidase (Biosource International, Camarillo, CA) at 1:200 dilution were used.

Inhibition assay: Antisera at appropriate dilution or mAb VK-9 at 0.1 $\mu g\,mL^{-1}$ were mixed with various concentrations of structurally related and unrelated carbohydrate antigens. The mixture was incubated overnight at 4 °C, and used in ELISA assays as described above. Percentage inhibition was calculated as the difference in absorbance between the uninhibited and inhibited serum.

ELISAs were also performed with sera that had been inhibited (absorbed) by incubation with MCF-7 or SK-MEL-28 cells. For this assay $17 \times 10^6$ cells were incubated with sera for 1 h, and the cells removed by centrifugation. ELISA was performed as described above.

Fluorescence-activated cell sorter (FACS) assay: FACS analyses were performed as previously described[8, 16] using FACS Scan (Becton-Dickinson, CA). Cells from the globo H-positive breast cancer cell line MCF-7 or the globo H-negative melanoma cell line SK-Mel-28 served as targets. An aliquot of 20 μL of diluted (1:20) antisera or mAb MBr1 and 20 μL of 1:30 goat anti-human IgM or IgG-labeled with fluorescein-isothiocyanate (FITC) (Southern Biotechnology Associates, Inc., Birmingham, AL) was used per $2 \times 10^5$ cells.

Complement-dependent cytotoxicity (CDC): Complement-dependent cytotoxicity was assayed at a serum dilution of 1:10 with MCF-7 cells and human complement by chromium-release assay as previously described.[16] All assays were performed in triplicates. Controls included cells incubated only with culture medium, complement, antisera, or mAb MBr1. Spontaneous release was the chromium released by target cells incubated with complement alone. The maximum release was the amount of $^{51}Cr$ released from target cells lysed with 1% Triton-100. Percent cytolysis was calculated according to the formula: specific release (%) = (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100.

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