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Evaluation of human antibody responses to keyhole limpet hemocyanin (KLH) on a carbohydrate microarray

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Abstract

Purpose—Keyhole limpet hemocyanin (KLH) is used as a vaccine adjuvant, as a carrier protein for small haptens, and as a treatment for bladder cancer. Immunization with KLH produces antibodies to tumor-associated carbohydrate antigens in animals, and these antibodies have been postulated as the basis of efficacy for bladder cancer treatment. The purpose of this study was to evaluate antibody responses to KLH in humans.

Experimental Design—A carbohydrate microarray was used to profile antibody responses in 14 individuals immunized with KLH plus alum adjuvant.

Results—8/14 individuals produced antibodies to at least one tumor-associated carbohydrate antigen. Increases to Lewis X, Lewis Y, GA1di, GM3, and sialyl Lewis A were observed in certain individuals, but, in general, antibody profiles were highly variable. Pre-immunization antibody levels to a subset of array antigens had a statistically significant correlation with the magnitude of the antibody response to KLH.

Conclusions and Clinical Relevance—Antibodies to tumor-associated carbohydrate antigens can be produced in humans, but antibody profiles differ considerably from person to person, which may contribute to variable clinical responses with KLH. Pre-treatment antibody levels to certain antigens may be useful for predicting which patients will respond favorably to KLH.

Keywords

Antibody; antigen array; glycan array; keyhole limpet hemocyanin; tumor-associated carbohydrate antigen

Introduction

Keyhole limpet hemocyanin (KLH) is large molecular weight glycoprotein of marine origin that is used extensively for basic research and clinical applications [1]. As a result of its high immunogenicity and low toxicity, KLH has become the standard carrier protein for the production of monoclonal and polyclonal antibodies to small molecule haptens such as peptides and oligosaccharides. In addition to numerous examples in basic research, several peptide-KLH conjugates and carbohydrate-KLH conjugates have progressed into clinical trials as cancer vaccines [2-5]. KLH has also been used for decades to assess the immune

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Conflict of Interest Statement
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status and performance of patients [6]. During a study on the immune competence of bladder cancer patients in the early 1970s, KLH injection was found to reduce the rate of tumor recurrence from 70% in the control patients to 11% in the KLH-treated group [7]. After many years of research and development, KLH is now clinically approved in The Netherlands, Austria, and South Korea for the prevention of recurrence of bladder cancer after surgery. In addition, KLH has entered late stage clinical trials in Europe and the United States [8, 9]. KLH is also used as an adjuvant, as a model glycoprotein, and for the development of vaccines against and clinical diagnosis of schistosomiasis due to cross-reactivity of infection sera with the glycoprotein [10, 11].

While KLH is a useful anticancer immunotherapeutic, clinical responses are only achieved in a subset of patients. For example, in one phase I/II clinical trial for the treatment of superficial bladder cancer, 30% of patients with refractory transitional cell carcinoma had complete remission while 50% of patients with carcinoma in situ had a complete response [12]. Information about the mechanism of action could be used to optimize its use, identify biomarkers for selecting patients that will benefit from KLH treatment, and monitor patients' progress. At present, however, the exact mechanism of action is not well understood and is still a matter of debate. Although KLH has modest anti-cancer activity on its own [13-15], it is widely believed that KLH acts via stimulation of the immune system [1, 16, 17]. Several mechanisms have been postulated including nonspecific immune stimulation [18], stimulation of cytotoxic T cells, and induction of anti-tumor antibodies. In support of the third mechanism, immunization of non-human animals with KLH has been shown to produce antibodies that bind tumor associated carbohydrate antigens including TF, Lewis Y, and GD3 [19, 20]. Studies on bladder cancer patients have shown an increase in anti-KLH antibody responses in patients responding to treatment [21]. Taken together, these results suggest that antibody profiles may be useful as biomarkers for tracking responses to KLH treatment.

Detailed evaluation of antibody responses to KLH is challenging. KLH is a complex glycoprotein with a wide variety of epitopes that could be targeted by the immune system. Although the immunodominant epitopes in humans are not known, the carbohydrate moieties of KLH are thought to be important antigenic determinants [22]. Carbohydrate-binding antibodies have historically been an especially difficult class of proteins to study due the lack of high-throughput methods for measuring carbohydrate-protein interactions. In recent years, carbohydrate antigen arrays have emerged as powerful tools for rapid analysis of carbohydrate-antibody binding [23-26]. Carbohydrate arrays contain many different glycans immobilized on a solid support in a spatially defined arrangement and enable evaluation of binding of lectins, antibodies, viruses, and cells to a large number of potential carbohydrate ligands. More important, when compared to traditional methods, analysis on a carbohydrate array can afford a broader view of carbohydrate-protein recognition with minimal effort, time, and material.

In this paper, a carbohydrate antigen array was used to compare antibody profiles of 14 individuals before and after immunization with keyhole limpet hemocyanin combined with alum adjuvant. We show that (a) a large proportion of the antibody response in humans is directed toward carbohydrate epitopes, (b) the specificity of the antibody response is different for different individuals, and (c) a subset of individuals produce antibodies to tumor associated carbohydrate antigens, including some carbohydrate antigens that have not been previously associated with the humoral response to KLH. Interestingly, we found that certain pre-immune serum antibody levels correlated with the magnitude of the KLH response, indicating that they may serve as biomarkers for selecting optimal patients prior to KLH treatment.

Materials and Methods

1.1 Serum samples

Serum samples were a gift from Dr. Monika Fleshner (University of Colorado, Boulder). Details describing the subject group and immunization procedure have been published [27]. Serum had been obtained from healthy men divided into two age groups [young (aged 20-35) and older (aged 65-79)], and two levels of physical activity (sedentary and active). The 14 samples included in this study consisted of 4 young and active, 2 young and sedentary, 4 older and active, and 4 older and sedentary individuals (for details, see Table S1 in the Supporting Information). KLH was adsorbed onto alum adjuvant and injected intramuscularly. Sera from day 0 (baseline, pre-immune) and day 21 (post-immune) were used in this study.

1.2 Evaluation of anti-carbohydrate antibodies in KLH antisera

1.2.1 Serum antibody binding to KLH vs. oxidized KLH—All steps were performed at room temperature. Oxidized KLH (oxKLH) was prepared according to the procedure of Woodward et al. [28]. Microtiter plate wells (Nunc, Flat Bottom, MaxiSorp, #436110) were coated with 500 ng/well KLH (Sigma-Aldrich) in 50mM carbonate buffer, pH 9.6, for 1.5 h. Wells were washed 5 × 200 μL PBST0.05 (1X PBS containing 0.05% v/v Tween 20), then once with 50 mM sodium acetate buffer, pH 4.4. Wells were incubated with sodium periodate (20mM in sodium acetate buffer, 100 μl/well) or KLH alone and allowed to stand in the dark for 1 h. Wells containing sodium periodate were washed once with sodium acetate buffer, and then incubated with freshly prepared sodium borohydride (50 mM in PBS, 100 μL/well) for 0.5 h. Wells were washed 5 × 200 μL PBST0.05 and blocked with 3% BSA/PBS for 1 h. Sera were added to wells at a dilution of 1:100 in PBS and incubated for 1 h. Wells were washed with 4 × 200 μL of PBST0.05. Next, alkaline-phosphatase conjugated goat anti-human IgA + IgG + IgM (H+L) (600 ng/mL in 3% BSA/PBS) was added (100 μL/well). After 0.5 h, wells were washed 7 × 200 μL of PBST0.05, then once with water. Enzyme substrate 4-methylumbelliferyl phosphate in Tris buffer, pH 9.0, was added and fluorescence was measured with an FLx800 Multi-Detection Microplate Reader (BioTek Instruments, Vermont) at 460 nm.

1.2.2 Saccharide inhibition of anti-KLH binding—Microtiter plate wells were coated with 500 ng/well KLH in 50 mM carbonate buffer (pH 9.6) overnight at 4 °C. All subsequent steps were performed at room temperature. Plates were blocked with 3% BSA/PBS (w/v) for 1 h. Sera were diluted to 1:100 in 1X PBS and pre-incubated for 30 min with or without (control) a cocktail containing 100 mM each of methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, β-lactose, D-cellobiose, D-maltose monohydrate, L-fucose, N-acetyl-D-galactosamine, and N-acetyl-D-glucosamine (Sigma-Aldrich). Samples (100 μL/well) were added to the KLH coated wells and incubated for 2 h. Wells were washed with 4 × 200 μL of PBST0.05. Next, alkaline-phosphatase conjugated goat anti-human IgA + IgG + IgM (H+L) (600 ng/mL in 3% BSA/PBS) was added (100 μL/well). After 0.5 h, wells were washed 7 × 200 μL of PBST0.05, then once with water. Enzyme substrate 4-methylumbelliferyl phosphate in Tris buffer, pH 9.0, was added and fluorescence was measured with an FLx800 Multi-Detection Microplate Reader at 460 nm. Results are the mean of duplicate experiments.

1.3 Fabrication of carbohydrate microarray and analysis of serum samples

Carbohydrate microarrays were fabricated as reported previously [29]. Each of the 114 components was printed in duplicate in a 20 × 12 grid of 110 μm diameter spots. 16

complete arrays were printed on each slide. A full description of array components is given in Table S3 (see Supporting Information). Printed slides were stored at -20 °C until use. Slides were assembled on 16-well slide holders and blocked with 3% BSA/PBS overnight at 4 °C, then washed 6 × 200 μL PBST0.05. Serum samples were dilution to 1:50 in 3% BSA/PBST0.05, added to arrays (100 μL/well), covered with a seal strip, and allowed to incubate with gentle shaking for 4 h at 37 °C. Each sample was analyzed in triplicate. After washing with 3 × 200 μL PBST0.05, detection of bound antibodies was carried out by incubating with Cy3-conjugated goat anti-human IgA + IgG + IgM (H+L) (Jackson ImmunoResearch Laboratories, Inc.) in 3% HSA/1% BSA/PBS (2 μg/mL; 100 μL/well) at 37 °C. After 2 h, slides were washed 7 × 200 μL PBST0.05, removed from holders, immersed in wash buffer for 5 min then centrifuged at 453*g* for 5 min.

Slides were scanned at 10 μm resolution with a Genepix 4000B microarray scanner (Molecular Devices Corporation, Union City, CA). Image analysis was carried out with Genepix Pro 6.0 analysis software (Molecular Devices Corporation). Spots were defined as circular features with a maximum diameter of 100 μm. Features were allowed to be resized as far as 70 μm as needed. Local background subtraction (median background) was performed. Initial data processing was performed with Microsoft Excel. The background-subtracted median pixel intensity feature was used for all analyses. Intensities above 50000 were corrected using the algorithm of Lyng et al. [30]. The final reported values for an experiment are the average signal from triplicate slides (6 spots).

To identify statistically significant differences between pre- and post-immune sera, we used results from our previous longitudinal study on technical and intra-individual variability of anti-carbohydrate antibody profiles [29]. To be significant, changes should be larger than expected based on the technical variability of our assay as well as the natural variability that occurs over time in un-immunized individuals. In the previous study, sera were collected from 7 healthy donors over a period ranging from 3 to 13 weeks. We calculated the average variance across all carbohydrates, $\text{var}_{\text{collection}}$, for the 7 donors. The between-collection standard deviation estimate, SD, was, in turn, calculated from the variance: $\text{SD}_{\text{collection}} = \sqrt{\text{var}_{collection}} = 0.298 \quad (\log \text{ base } \quad 2)$. If one considers the difference between signals from any two samples from the same individual collected at different time points and run on the same batch of slides, the variance of the difference should be $2 \times \text{var}_{\text{collection}}$. Therefore, the standard deviation of a collection-to-collection difference should be $\sqrt{2} \times \text{SD}_{\text{collection}} = \sqrt{2} \times 0.298 = 0.421$. Using a false positive threshold rate of 0.1% ($p < 0.001$), we can flag as significant those differences larger than 0.421 × 3.29 = 1.39; or 2.61 fold, in normal scale. Correlations between pre-immune antibody levels and the response to KLH were evaluated using Partek Genomics Suite software using the Pearson correlation function.

Results

To evaluate the antibody responses to KLH, a series of experiments were carried out on human sera before and after immunization obtained from a previous study carried out by the Fleshner group [27]. We note that the volunteers in the Fleshner study were immunized with KLH plus alum. Alum adjuvant is not immunogenic on its own and does not act as a hapten [31]. However, alum can affect the magnitude and specificity of the antibody response to adsorbed antigens [32]. Therefore, the results and observation presented in this report are for the combination of KLH plus alum.

1.4 Evaluation of anti-carbohydrate antibodies in KLH antisera

Before embarking on an in depth analysis of the anti-carbohydrate responses, we first determined whether the majority of the antibody response to KLH plus alum in humans was indeed targeting carbohydrate epitopes by using two enzyme-linked immunosorbent assay (ELISA)-based approaches. First, we compared antibody binding to KLH with periodate-oxidized KLH (oxKLH). Mild treatment of glycoproteins with periodate cleaves vicinal cis diols of glycans but does not affect the peptide backbone [28]; therefore, oxidation should abolish recognition of most, but not all, carbohydrate epitopes. The level of induced antibody was calculated as the difference between the signals obtained for the post- and the pre-immune serum. In cases where the pre-immune signal was higher than post-immune signal, the difference was set to zero. For all 14 subjects, the amount of KLH-induced antibodies that bound to oxKLH was about 33% to 100% lower than the amount bound to KLH (Figure 1a), which suggests that cleavage of carbohydrate residues on KLH substantially affects recognition of KLH-induced antibodies and that for most individuals, the majority of the antibody response to KLH plus alum is directed at the carbohydrate epitopes.

In the second ELISA approach, we determined whether binding of KLH could be competitively inhibited by a cocktail containing 100 mM each of methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, β-lactose, D-cellobiose, D-maltose monohydrate, L-fucose, N-acetyl-D-galactosamine, and N-acetyl-D-glucosamine. Inhibition of induced antibody binding by exogenous sugar cocktail ranged from 27 to 100%, with a median of 74% (Figure 1b).

Taken together, results from the two experiments show that the human antibody response to KLH primarily targets the carbohydrate epitopes on KLH and justify the use of a carbohydrate antigen array, rather than a peptide array, for further characterization of the anti-KLH response.

1.5 Analysis of KLH antisera on a carbohydrate microarray

We have previously reported the development and applications of a carbohydrate microarray [29, 33-40]. In the present study, samples were profiled on a version of the carbohydrate antigen array comprising 107 components including 78 glycoconjugates, 24 glycoproteins, and 5 controls (see Table S3 in Supporting Information). The glycoconjugates are made up of structurally defined homogenous carbohydrates, which are attached to BSA or HSA. Serum incubation was done at 37 °C to match normal human physiological temperature.

Differences observed between pre- and post-immune microarray profiles could be due to technical variability, natural biological variability over time, or immunization. To identify those changes that were beyond technical and natural variability, we utilized data from a previous study where we evaluated the temporal variation of anti-carbohydrate antibodies in 7 healthy donors over a period ranging from 3 to 13 weeks [29]. Based on that study, we determined that the largest difference beyond experimental and natural biological variability that one might expect to see between two serum samples that were collected from the same individual at different time points and analyzed in the same experiment was about 2.6-fold (or 1.39 in log base 2; for p=0.001; see Materials and Methods for details). Therefore, for the current study, differences between pre- and post-immune sera larger than about 2.6-fold were considered statistically significant.

1.5.1 Changes in anti-KLH antibodies post-immunization—Changes in antibody profiles are shown in Figure 2. We found that 10 subjects showed significant, yet variable, increases in antibody levels to KLH in post-immune sera. Four subjects showed no

significant response to KLH. These results are consistent with a previous study showing that some individuals do not mount an appreciable antibody response to KLH [41] and are consistent with the Fleshner study on a group basis. Fleshner et al. evaluated the antibody response to KLH and reported mean values for the each of 4 groups of volunteers (See Table S1 in Supporting Information for group descriptions). They showed that anti-KLH IgG and IgM serum concentrations increased for all subgroups and significant correlations with physical activity and age, respectively. We also observed average increases in anti-KLH signal on the array for all 4 groups. Individual data from the Fleshner study was not available for comparison. Differences in antibody signals for KLH and oxKLH measured on the array were consistent with the results obtained by ELISA. The increase in the anti-KLH signal in the 8 subjects who had a response ranged from 3.9- to 13.8-fold. In contrast, no significant changes were observed for oxKLH.

1.5.2 Changes in other carbohydrate antibody levels post-immunization—Next, we analyzed changes to individual glycans. The carbohydrates on KLH have not been fully characterized but several structures including Galβ1-6Man [42], Galβ1-3GalNAc [19], Fucα1-3GalNAcα [43], and Galβ1-4Galβ1-4Fucα1-6 core modifications to the reducing end GlcNAc residue of N-linked glycans [44] have been identified. Several of these as well as many other glycans were present on the array (see Figure 2). In general, we found considerable variation from person-to-person in the response to carbohydrate structures (Figure 2). Some individuals, for example subjects 1, 3, 9, 11, and 13, showed highly focused responses to a relatively small number of antigens. Other individuals, such as subjects 2, 7, and 12, showed significant changes to a larger number of epitopes. Responses to individual epitopes did not necessarily correlate with overall responses to KLH. In particular, individuals having the same signal to KLH had different antibody profiles; thus the magnitude of the response to KLH could not have been used to predict the overall profile or the specific responses to individual carbohydrate antigens. For example, subject 5 had a 10.2-fold increase (3.19 on log base 2 scale) to KLH and a 9.1-fold increase to Lewis Y, while subject 6, who had an 12.0-fold increase in anti-KLH antibodies, had only a 2.6-fold increase in anti-Lewis Y antibodies. For these two individuals, the absolute responses to KLH were also of similar magnitude.

1.5.3 Responses to tumor-associated carbohydrate antigens—Classification of a carbohydrate as a tumor antigen is somewhat subjective; therefore, we used a broad interpretation of the term to include any carbohydrate that has been reported to have increased expression on tumors and/or tumor cell lines. Altered expression of carbohydrate antigens in bladder cancer has been recently reviewed [45]. Lewis X and Sialyl Lewis X are highly expressed on bladder tumors and expression correlates with a poor prognosis. Other antigens expressed on bladder tumors include Tn, TF, Sialyl-TF, Lewis Y, and GM3. Many known tumor-associated carbohydrate antigens, however, have not been studied in the context of bladder tumors. For the purposes of this analysis, antibody increases to these antigens were also included as anti-tumor antigen responses. Notable tumor antigens on the array include Tn, Lewis Y, Lewis X, dimeric Lewis X, Sialyl Lewis A, Sialyl Lewis X, Gb3, GM3, GA1, and the LNT trisaccharide [45-47]. We found that a subset of individuals produced antibodies that bound known carbohydrate tumor antigens on the array. Of the 14 subjects, 4 had significant increases in response to Lewis Y and one subject had a decrease in signal. Subject 5 had an 8- and 10-fold increase in signal to Lewis Y and dimeric Lewis X, respectively. Wirguin et al. reported that KLH immunization in rats induces antibodies that recognize the TF antigen (Galβ1-3GalNAcα) and its β-anomer (GA1di; Galβ1-3GalNAcβ) [19]. Although it is not known whether the same response would be expected or observed in humans, we observe that 3 subjects had significant increases in signal to GA1di. In contrast to the Wirguin study, we observed only minimal responses to

asialo-fetuin and asialo-glycophorin, two glycoproteins that are known to present the TF epitope. Loo et al. reported results of a preclinical study of a potent cytotoxic monoclonal antibody that recognizes an N-linked carbohydrate expressed on more than 90% of colorectal, gastric, and pancreatic tumors [48]. The minimal antibody binding epitope was determined to be Galβ1-3GlcNAcβ1-3Gal. In our analysis, 3 individuals had significant responses to LNT (Galβ1-3GlcNAcβ1-3Galβ). Other tumor associated carbohydrates with significant responses include GM3 [49, 50] and Sialyl Lewis A (CA 19-9) [51, 52], each of which had 3 subjects having significant increases in antibody levels.

1.6 Relationships between array profiles and total IgG and IgM

Several individuals had increases in antibody levels to many carbohydrates on the array. This type of response could be the result of an increase in overall antibody levels. To gain more insight into this possibility, we measured total IgG and IgM concentrations for each of the 14 volunteers in our study. We found that changes in total IgG and IgM did not necessarily relate to changes observed on the array (see Figure 2b). For example, subject 8 had the largest increase in total IgG (4.6) and the second largest increase in IgM (3.1) but showed only a small number of changes on the array. Total IgG and IgM changes also did not correlate with antibody responses to KLH. For example, subject 1 had a 12 fold increase to KLH but had little or no change in total IgG and IgM.

1.7 Correlations between pre-immune antibody levels and the magnitude of the KLH response

Next, we evaluated whether pre-immunization serum antibody levels correlated with the magnitude of the antibody response to KLH. We found no statistically significant correlation between total IgG or total IgM levels and the response to KLH; however, statistically significant correlations ($p < 0.05$) were observed for 13 array components: GlcNAca1-4Galb-BSA, asialofetuin, LSTc-BSA, KLH, asialo-OSM, ovalbumin, thyroglobulin, heat shock protein 90, lactose-BSA, P1-BSA, GA1di-BSA, prostate specific antigen, and BG-B-BSA (Dextra). For all 13, lower antibody levels correlated with a larger anti-KLH response. The top six (GlcNAca1-4Galb-BSA, asialo-fetuin, LSTc-BSA, KLH, asialo-OSM, ovalbumin) had p values <0.02. In an effort to improve the correlations, we averaged the antibody levels across these six antigens to provide a single value and plotted those values versus the KLH response for each individual (see Figure 4). The correlation between the average and the antibody response to KLH was highly statistically significant ($p = 0.000073$).

Discussion

KLH is a glycoprotein used extensively in basic research as a carrier protein and as a test antigen for evaluating immune competence. In addition, it is used clinically as an immunotherapeutic agent for the prevention of recurrence of bladder cancer. While KLH treatment can be highly effective, only a subset of cancer patients achieves a clinical response. To improve clinical use of KLH, it is desirable to a) develop methods to predict which patients will respond favorably to KLH treatment, and b) identify biomarkers that indicate, preferably at an early stage of treatment, if a particular patient receiving KLH treatment is responding in a beneficial manner. In addition, there has been considerable interest in understanding the mechanism of action to aid our understanding of immunology and facilitate the identification of biomarkers.

Antibodies are a key element of the immune response to KLH and antibody responses could contribute to the mechanism of action of KLH as an immunotherapeutic for cancer. While the role of antibodies has been well appreciated, they have been difficult to evaluate using traditional methods. In this study, we used a carbohydrate microarray to profile the

repertoire of antibody responses induced by KLH plus alum adjuvant in human subjects. Our results provide the most comprehensive evaluation of anti-carbohydrate antibody responses published to date and have implications for basic and clinical research on KLH.

First, our results have implications for studies on the mechanism of action of KLH. Although there have been a number of interesting studies on KLH, the mechanism of action is still a matter of debate. Mechanisms involving general immune stimulation, induction of cellular immunity, and induction of specific antibodies have been postulated [53]. Additional information regarding the mechanism could be useful for basic research and for developing biomarkers to aid in the clinical applications of KLH. In support of antibodies, previous studies in mice and rats have shown that immunization with KLH can induce antibodies to tumor associated carbohydrate antigens (TACAs). For this mechanism to operate in humans, however, responses to these or other tumor antigens must be produced. In this study, we show that the majority of the antibody responses in humans are directed at the carbohydrate epitopes. In addition, we show for the first time that antibodies to TACAs are induced in humans after immunization with KLH plus alum adjuvant. Eight of 14 individuals produced antibodies to at least one tumor antigen. The targeted TACAs included ones previously reported as well as some not previously observed as a result of KLH immunization. The ability to detect previously unrecognized epitopes is one of the advantages of using an array format for the analysis. In addition to individual antigens, the carbohydrate microarray also provided a more global view of responses. Some subjects had focused responses towards a small subset of carbohydrates while other individuals had antibody responses to a multitude of carbohydrate epitopes, resembling a general immunostimulatory effect. For example, subjects 2 and 7 had increased antibody levels to a multitude of glycans. While these types of responses could be the result of changes in total antibody levels, they did not correlate with total IgG or IgM levels.

Second, the results described in this study also have implications for clinical implementation of KLH as an immunotherapeutic for cancer. First, we found that antibody levels to certain antigens prior to immunization are inversely correlated with the magnitude of the response to KLH. By taking the average of the antibody levels to the top six antigens, a highly statistically significant correlation is obtained ($p < 0.00007$). Previous studies have shown that antibody responses to KLH correlate with clinical responses [21]. Therefore, pre-immune antibody levels to these antigens may serve as biomarkers for selecting appropriate patients for KLH treatment. For example, one might select patients with below average antibody levels to the antigens for KLH treatment. Although additional studies are necessary to validate this discovery and optimize the method, these initial results are remarkable. Second, the microarray also provides a tool for monitoring responses during treatment. The optimal immunological response is not yet known. Our array results show that there are significant differences in responses to specific glycans as well as major differences in overall responses between individuals. These differences could contribute to the differences in clinical outcomes, but additional studies will be needed to evaluate this possibility. While these variations would be difficult to detect using traditional methods, our results show that the carbohydrate microarray is a highly effective tool for rapidly measuring these responses and distinguishing different types of immune responses to KLH.

The results presented here also have ramifications for the use of KLH as a carrier protein in vaccine research and development. First, KLH conjugates of tumor associated carbohydrate antigens, including Tn, GM3, and Lewis Y, have been synthesized and used in basic and clinical cancer vaccine research. We found that KLH itself can induce antibodies to some of these antigens in some individuals; therefore, it is important to consider, when analyzing the efficacy of a KLH conjugate vaccine candidate, (i) if KLH presents the antigen of interest, (ii) whether the responses to KLH are protective or nonprotective, and (iii) if KLH induces

antibodies that enhance or suppress the response to the carbohydrate antigen of interest. The information obtained about KLH may be important for other glycosylated proteins used as carrier proteins, such as ovalbumin.

Finally, the results described in this study illustrate the utility of carbohydrate microarrays for evaluating immune responses to large, complex immunogens. A number of other glycoproteins, cells, and viruses/virus-like particles are in development as vaccines. Our results with KLH indicate that immune responses to carbohydrate antigens may represent a major component of the immune response generated with these immunogens and that responses to the carbohydrates may vary significantly from person to person. The array provides a useful tool for evaluating these responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Fuc	fucose
Gal	galactose
GalNAc	*N*-acetylgalactosamine
Glc	glucose
GlcNAc	*N*-acetylglucosamine
KLH	keyhole limpet hemocyanin
Man	mannose
oxKLH	periodate oxidized keyhole limpet hemocyanin
TACA	tumor-associated carbohydrate antigen

References

1. Harris JR, Markl J. Keyhole limpet hemocyanin (KLH): a biomedical review. Micron. 1999; 30:597–623. [PubMed: 10544506]
2. Helling F, Zhang S, Shang A, Adluri S, et al. GM2-KLH Conjugate Vaccine: Increased Immunogenicity in Melanoma Patients after Administration with Immunological Adjuvant QS-21. Cancer Res. 1995; 55:2783. [PubMed: 7796403]
3. Gilewski TA, Ragupathi G, Dickler M, Powell S, et al. Immunization of high-risk breast cancer patients with clustered sTn-KLH conjugate plus the immunologic adjuvant QS-21. Clin. Cancer Res. 2007; 13:2977–2985. [PubMed: 17504999]
4. Slovin SF, Ragupathi G, Musselli C, Olkiewicz K, et al. Fully synthetic carbohydrate-based vaccines in biochemically relapsed prostate cancer: Clinical trial results with alpha-*N*-acetylgalactosamine-*O*-Serine/Threonine conjugate vaccine. J. Clin. Oncol. 2003; 21:4292–4298. [PubMed: 14645418]
5. Schumacher K. Keyhole limpet hemocyanin (KLH) conjugate vaccines as novel therapeutic tools in malignant disorders. J. Cancer Res. Clin. Oncol. 2001; 127(Suppl 2):R1–2. [PubMed: 11768617]

6. Spatz M, Eibl N, Hink S, Wolf HM, et al. Impaired primary immune response in type-1 diabetes. Functional impairment at the level of APCs and T-cells. Cell. Immunol. 2003; 221:15–26. [PubMed: 12742378]
7. Olsson CA, Chute R, Rao CN. Immunologic reduction of bladder cancer recurrence rate. J. Urol. 1974; 111:173–176. [PubMed: 4810758]
8. Lamm DL, Dehaven JI, Riggs DR. Keyhole limpet hemocyanin immunotherapy of bladder cancer: Laboratory and clinical studies. Eur. Urol. 2000; 37:41–44. [PubMed: 10828686]
9. Jurincic-Winkler CD, Metz KA, Beuth J, Klippel KF. Keyhole limpet hemocyanin for carcinoma in situ of the bladder: A long-term follow-up study. Eur. Urol. 2000; 37:45–49. [PubMed: 10828687]
10. Grzych JM, Dissous C, Capron M, Torres S, et al. Schistosoma mansoni shares a protective carbohydrate epitope with keyhole limpet hemocyanin. J. Exp. Med. 1987; 165:865–878. [PubMed: 2434601]
11. Zhang SL, CordonCardo C, Zhang HS, Reuter VE, et al. Selection of tumor antigens as targets for immune attack using immunohistochemistry .1. Focus on gangliosides. Int. J. Cancer. 1997; 73:42–49. [PubMed: 9334808]
12. Lamm DL. Laboratory and clinical experience with keyhole limpet hemocyanin (immucothelÂ®) in superficial bladder cancer. J. Urol. Urogynäkol. 2003; 10:18–21.
13. Riggs DR, Jackson B, Vona-Davis L, McFadden D. In vitro anticancer effects of a novel immunostimulant: Keyhole limpet hemocyanin. J. Surgical Res. 2002; 108:279–284.
14. Riggs DR, Jackson BJ, Vona-Davis L, Nigam A, McFadden DW. In vitro effects of keyhole limpet hemocyanin in breast and pancreatic cancer in regards to cell growth, cytokine production, and apoptosis. Am. J. Surg. 2005; 189:680–684. [PubMed: 15910720]
15. McFadden DW, Riggs DR, Jackson BJ, Vona-Davis L. Keyhole limpet hemocyanin, a novel immune stimulant with promising anticancer activity in Barrett's esophageal adenocarcinoma. Am. J. Surg. 2003; 186:552–555. [PubMed: 14599624]
16. Presicce P, Taddeo A, Conti A, Villa ML, Bella SD. Keyhole limpet hemocyanin induces the activation and maturation of human dendritic cells through the involvement of mannose receptor. Mol. Immunol. 2008; 45:1136–1145. [PubMed: 17765973]
17. Lamm DL, Dehaven JI, Riggs DR, Ebert RF. Immunotherapy Of Murine Bladder-Cancer With Keyhole Limpet Hemocyanin (Klh). J. Urol. 1993; 149:648–652. [PubMed: 8437283]
18. Tzianabos AO. Polysaccharide immunomodulators as therapeutic agents: structural aspects and biologic function. Clin. Microbiol. Rev. 2000; 13:523–533. [PubMed: 11023954]
19. Wirguin I, Suturkovamilosevic L, Briani C, Latov N. Keyhole Limpet Hemocyanin Contains Gal(Beta-1-3)-Galnac Determinants That Are Cross-Reactive With The T-Antigen. Cancer Immunol. Immunother. 1995; 40:307–310. [PubMed: 7600562]
20. May RJ, Beenhouwer DO, Scharff MD. Antibodies to Keyhole Limpet Hemocyanin Cross-React with an Epitope on the Polysaccharide Capsule of Cryptococcus neoformans and Other Carbohydrates: Implications for Vaccine Development. J. Immunol. 2003; 171:4905. [PubMed: 14568972]
21. Jurincic-Winkler CD, VonderKammer H, Beuth J, Scheit KH, Klippel KF. Antibody response to keyhole limpet hemocyanin (KLH) treatment in patients with superficial bladder carcinoma. Anticancer Res. 1996; 16:2105–2110. [PubMed: 8712751]
22. Van Kuik, JA.; Kamerling, JP.; Vliegenthart, JFG. Invertebrate Oxygen Carriers. Preaux, G.; Lontie, R., editors. Leuven University Press; Leuven: 1990. p. 157-163.
23. Park S, Lee M, Shin I. Carbohydrate microarrays as powerful tools in studies of carbohydrate-mediated biological processes. Chemical Communications. 2008:4389. [PubMed: 18802572]
24. Liang PH, Wu CY, Greenberg WA, Wong CH. Glycan arrays: biological and medical applications. Curr. Opin. Chem. Biol. 2008
25. Oyelaran O, Gildersleeve JC. Application of carbohydrate array technology to antigen discovery and vaccine development. Expert Rev. Vaccines. 2007; 6:957–969. [PubMed: 18377358]
26. Paulson JC, Blixt O, Collins BE. Sweet spots in functional glycomics. Nat. Chem. Biol. 2006; 2:238–248. [PubMed: 16619023]

27. Smith TP, Kennedy SL, Fleshner M. Influence of age and physical activity on the primary in vivo antibody and T cell-mediated responses in men. J. Appl. Physiol. 2004; 97:491–498. [PubMed: 15247195]
28. Woodward MP, Young WW, Bloodgood RA. Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. J. Immunol. Methods. 1985; 78:143–153. [PubMed: 2580026]
29. Oyelaran O, McShane LM, Dodd L, Gildersleeve JC. Profiling human serum antibodies with a carbohydrate antigen microarray. J. Proteome Res. 2009; 8:4301–4310. [PubMed: 19624168]
30. Lyng H, Badiee A, Svendsrud DH, Hovig E, et al. Profound influence of microarray scanner characteristics on gene expression ratios: Analysis and procedure for correction. BMC Genomics. 2004; 5
31. Nicklas W. Aluminum salts. Res. Immunol. 1992; 143:489–494. [PubMed: 1439128]
32. Lindblad EB. Aluminium adjuvants—in retrospect and prospect. Vaccine. 2004; 22:3658–3668. [PubMed: 15315845]
33. Oyelaran OO, Li Q, Farnsworth DF, Gildersleeve JC. Microarrays with Varying Carbohydrate Density Reveal Distinct Subpopulations of Serum Antibodies. J. Proteome Res. 2009; 8:3529–3538. [PubMed: 19366269]
34. Li Q, Anver MR, Butcher DO, Gildersleeve JC. Resolving Conflicting Data on Expression of the Tn Antigen and Implications for Clinical Trials with Cancer Vaccines. Mol. Cancer. Res. 2009; 8:971–979.
35. Hsu KL, Gildersleeve JC, Mahal LK. A simple strategy for the creation of a recombinant lectin microarray. Mol. BioSyst. 2008; 4:654–662. [PubMed: 18493664]
36. Gildersleeve JC, Oyelaran O, Simpson JT, Allred B. Improved procedure for Direct Coupling of Carbohydrates to Proteins via Reductive Amination. Bioconjugate Chem. 2008; 19:1485–1490.
37. Manimala JC, Roach TA, Li Z, Gildersleeve JC. High-throughput carbohydrate microarray profiling of 27 antibodies demonstrates widespread specificity problems. Glycobiology. 2007; 17:17C–23C.
38. Manimala JC, Roach TA, Li ZT, Gildersleeve JC. High-throughput carbohydrate microarray analysis of 24 lectins. Angew. Chem. Int. Ed. 2006; 45:3607–3610.
39. Manimala J, Li Z, Jain A, VedBrat S, Gildersleeve JC. Carbohydrate array analysis of anti-Tn antibodies and lectins reveals unexpected specificities: Implications for diagnostic and vaccine development. ChemBioChem. 2005; 6:2229–2241. [PubMed: 16252298]
40. Li Q, Anver MR, Li Z, Butcher DO, Gildersleeve JC. GalNAcα1-3Gal, a New Prognostic Marker for Cervical Cancer. Int. J. Cancer. 2009 in press.
41. Burke GP, Smith KA, Stocking RI, Ferm M, McIntyre OR. Anti-keyhole limpet hemocyanin antibody in normal unsensitized individuals. J. Allergy Clin. Immunol. 1977; 59:309–313. [PubMed: 850020]
42. Kurokawa T, Wuhrer M, Lochnit G, Geyer H, et al. Hemocyanin from the keyhole limpet Megathura crenulata (KLH) carries a novel type of N-glycans with Gal(beta1-6)Man-motifs. Eur. J. Biochem. 2002; 269:5459–5473. [PubMed: 12423344]
43. Kantelhardt SR, Wuhrer M, Dennis RD, Doenhoff MJ, et al. Fuc(alpha1-->3)GalNAc-: the major antigenic motif of Schistosoma mansoni glycolipids implicated in infection sera and keyhole-limpet haemocyanin cross-reactivity. Biochem. J. 2002; 366:217–223. [PubMed: 11996672]
44. Wuhrer M, Robijn ML, Koeleman CA, Balog CI, et al. A novel Gal(beta1-4)Gal(beta1-4)Fuc(alpha1-6)-core modification attached to the proximal N-acetylglucosamine of keyhole limpet haemocyanin (KLH) N-glycans. Biochem. J. 2004; 378:625–632. [PubMed: 14613482]
45. Ohyama C. Glycosylation in bladder cancer. int. J. Clin. Oncol. 2008; 13:308–313. [PubMed: 18704630]
46. Fuster MM, Esko JD. The sweet and sour of cancer: Glycans as novel therapeutic targets. Nat. Rev. Cancer. 2005; 5:526–542. [PubMed: 16069816]
47. Dube DH, Bertozzi CR. Glycans in cancer and inflammation. Potential for therapeutics and diagnostics. Nat. Rev. Drug Discovery. 2005; 4:477–488.

48. Itzkowitz SH, Bloom EJ, Lau TS, Kim YS. Mucin associated Tn and sialosyl-Tn antigen expression in colorectal polyps. Gut. 1992; 33:518–523. [PubMed: 1582597]

49. Amaya S, Sasaki M, Watanabe Y, Tsui WMS, et al. Expression of MUC1 and MUC2 and carbohydrate antigen Tn change during malignant transformation of biliary papillomatosis. Histopathol. 2001; 38:550–560.

50. Nores GA, Dohi T, Taniguchi M, Hakomori S. Density-dependent recognition of cell surface GM3 by a certain anti-melanoma antibody, and GM3 lactone as a possible immunogen: requirements for tumor-associated antigen and immunogen. J. Immunol. 1987; 139:3171–3176. [PubMed: 3668254]

51. Saito K, Fujii Y, Kawakami S, Hayashi T, et al. Increased expression of sialyl-Lewis A correlates with poor survival in upper urinary tract urothelial cancer patients. Anticancer Res. 2003; 23:3441–3446. [PubMed: 12926087]

52. Amano K, Hayashi S, Kubota T, Fujii N, Yokota S. Reactivities of Lewis antigen monoclonal antibodies with the lipopolysaccharides of Helicobacter pylori strains isolated from patients with gastroduodenal diseases in Japan. Clin. Diagn. Lab. Immunol. 1997; 4:540–544. [PubMed: 9302202]

53. Hecht ML, Rosental B, Horlacher T, Hershkovitz O, et al. Natural cytotoxicity receptors NKp30, NKp44 and NKp46 bind to different heparan sulfate/heparin sequences. J. Proteome Res. 2009; 8:712–720. [PubMed: 19196184]

Statement of clinical relevance

Keyhole limpet hemocyanin (KLH) is a clinically approved immunotherapeutic agent for bladder cancer. While KLH treatment has many advantages, such as very low toxicity, it is only effective in 30-50% of patients and there are no methods available for predicting which patients will respond. In this study, we show that the immuno-dominant epitopes on KLH are carbohydrates and that antibodies to tumor-associated carbohydrate antigens are produced in humans. In addition, we show that pre-existing antibody levels to a select subset of glycans correlate with the magnitude of the antibody response to KLH. Since previous studies on bladder cancer patients have shown that anti-KLH antibody responses correlate with clinical responses, measurement of those antibody levels prior to treatment may be useful for predicting which patients will respond favorably to KLH.

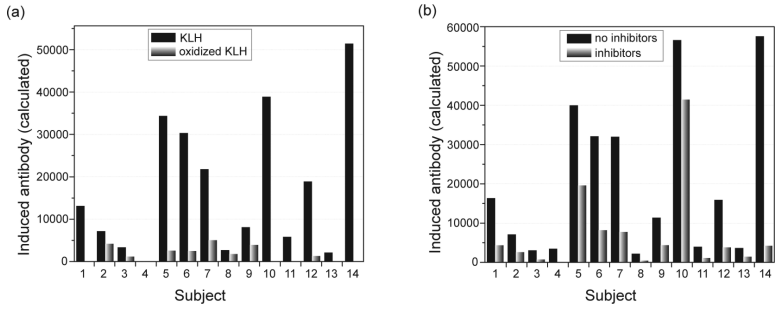

Figure 1. Proportion of antibody response targeting carbohydrates

(a) Comparison of serum antibody binding to KLH or periodate oxidized KLH, (b) comparison of serum antibody binding to KLH in the presence or absence an inhibitory cocktail of sugars (100 mM each of methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, β-lactose, D-cellobiose, D-maltose monohydrate, L-fucose, N-acetyl-D-galactosamine, and N-acetyl-D-glucosamine). The level of induced antibody was calculated as the difference in signal between pre-immune and post-immune serum samples.

Figure 2. Antibody responses to KLH plus alum

Changes in antibody profiles between preand post-immune sera measured on the carbohydrate microarray. Carbohydrates are indicated in the rows and subjects are indicated in the columns. Each rectangle represents the difference between the pre- and post-immune normalized signal [log-transformed (base 2)]. The color of the rectangle corresponds to values on the color scale to the left of the heat map. Gray rectangles represent no change or changes that were not statistically significant.

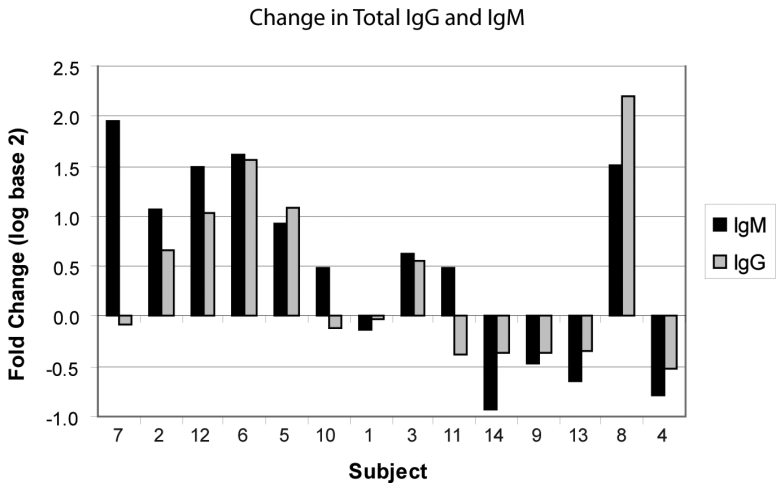

Figure 3. Changes in total antibody levels

Differences between pre- and post-immune sera for total IgG and IgM measured by ELISA. Each bar represents the ratio of the pre- and post-immune antibody concentrations, presented in log scale (base2).

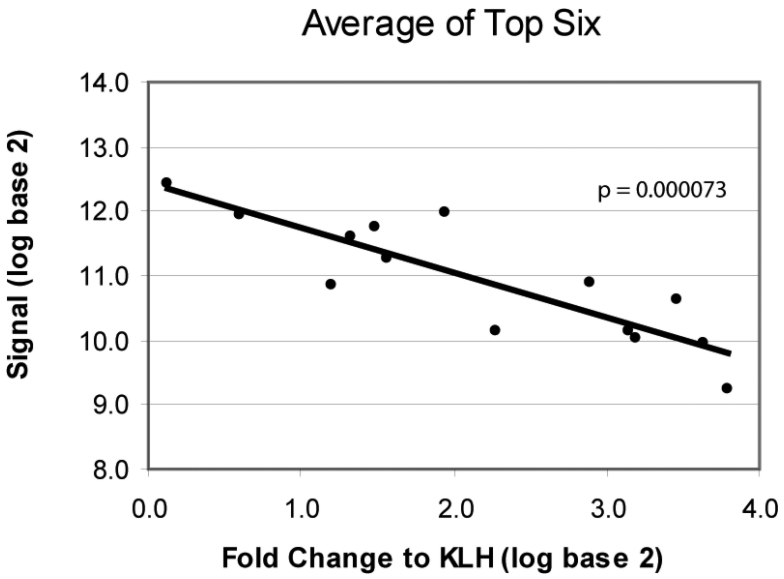

Figure 4. Correlations between pre-immune antibody levels and the KLH response

Pre-immune antibody signals to LSTc, GlcNAca1-3Gal, asialo-fetuin, KLH, ovalbumin, and asialo-OSM were averaged and the average value (log base 2) for each subject was plotted versus the magnitude of the antibody response to KLH (x-axis; log base 2) for that subject. The correlation was highly statistically significant ($p < 0.000073$).