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Serum Antibodies to Blood Group A Predict Survival on PROSTVAC-VF

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Abstract

Purpose—There is evidence that therapeutic cancer vaccines can lengthen survival for some cancer patients, but responses vary widely from one person to another. Methods to predict clinical outcomes will advance the field and provide new insights into critical determinants of *in vivo* efficacy.

Experimental Design—This retrospective study included 141 subjects from phase II trials of PROSTVAC-VF, a poxvirus based cancer vaccine currently in phase III clinical trials for advanced prostate cancer. A glycan microarray was used to profile pre-vaccination anti-glycan antibody populations in sera as potential biomarkers for PROSTVAC-VF. The screen for predictive biomarkers identified anti-glycan antibodies that consistently stratified subjects into groups with different Kaplan Meier survival estimates. Due to the potential for overfitting, a permutation test was used to estimate the false discovery rate.

Results—Pre-vaccination antibody levels to blood group A trisaccharide (BG-A_{tri}) were found to have a statistically significant correlation with survival. Long-term survival was approximately doubled in subjects with abundant anti-BG-A_{tri} IgM relative to subjects with little or no pre-existing IgM for BG-A_{tri}. This survival correlation was specific to vaccine treatment, as no correlation was observed in control patients immunized with wild-type poxviruses lacking the key tumor antigen, prostate specific antigen (PSA). Moreover, anti-BG-A_{tri} IgM levels were not correlated with general measures of disease severity, such as PSA levels, Gleason score, or Halabi predicted survival.

Conclusion—In addition to reporting a new potentially predictive biomarker for PROSTVAC-VF, this study highlights the utility of glycan microarray technology for improving our understanding of vaccine immunology.

Keywords

cancer vaccine; immunotherapy; biomarker; PROSTVAC-VF; glycan microarray

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Disclosure of Potential Conflicts of Interest

PROSTVAC-VF is being developed under a Cooperative Research and Development Agreement (CRADA) between Bovarian Nordic and the Center for Cancer Research (CCR). Drs. Gildersleeve, Campbell, Oyelaran, Schlom, and Gulley are co-inventors on a patent application covering the new biomarker reported in this manuscript.

Introduction

Personalized medicine bases treatment decisions on characteristics of individual patients and their diseases (1). One particular area set to benefit from personalized medicine is cancer vaccine therapy (2), which trains the immune system to recognize and eliminate malignant cells. While numerous clinical trials have been conducted and one therapeutic cancer vaccine has earned FDA approval, clinical responses vary widely from one patient to another. Certain patients achieve long lasting responses while others appear to receive little or no benefit. A longstanding objective in the field is to devise methods to predict a patient's benefit from cancer vaccine therapy. By targeting treatment to likely responders, a predictive method can significantly improve clinical efficacy while simultaneously reducing health care costs.

Methods to optimize treatment decisions generally fall into two categories: (1) assessing immune responses after vaccination and (2) predicting clinical outcomes prior to vaccination. A number of studies have identified survival associations with immune responses measured after vaccination. Potential biomarkers [reviewed in (3–5)] include T-cell responses, delayed type hypersensitivity, immunosuppressive regulatory T-cells, cytokine profiles, humoral responses, anti-vector titers, and circulating tumor cells. While promising, none of these approaches have been fully validated. Moreover, these methods require treatment of patients for at least several months before assessing response. Methods to predict outcomes prior to treatment can avoid unnecessary side effects in patients unlikely to benefit from the vaccine as well as allow patients to pursue alternative treatments at an earlier time point in the course of their disease. At present, however, the factors that predispose an individual to have a productive response to a vaccine, thus leading to improved survival, are unknown.

Pre-existing antibodies potentially influence clinical outcomes of patients treated with a cancer vaccine in several ways. Numerous anti-tumor autoantibodies have been reported (6, 7), and these antibodies may induce antibody dependent cell-mediated cytotoxicity, block metastasis, or directly kill tumors. These antibodies could act synergistically with vaccine-induced responses leading to improved survival. Additionally, pre-existing humoral immunity may augment vaccine responses by enhancing cross-presentation (8) and antigen spreading (9). Alternatively, other pre-existing antibodies may promote tumor growth antagonizing vaccine-induced responses. Fourth, pre-existing antibodies to vaccine components could affect immune responses induced by vaccination (10). While pre-existing antibodies could influence vaccine efficacy in many ways, most studies have shown no correlation, and the few reported correlations were modest trends towards improved survival or disease stabilization in a small number of subjects (11, 12).

Prior studies on relationships between serum antibodies and survival have primarily focused on antibodies that recognize proteins and/or peptides (13, 14). Carbohydrates are an equally important class of antigens, and many cancer vaccines currently in clinical trials, such as viral vector vaccines, whole cell vaccines, glycoprotein-based vaccines, and carbohydrate-based vaccines, possess carbohydrates that could be targeted by the immune system. Abnormal glycosylation is a hallmark of malignancy (15, 16), and many of the glycans on these vaccines, as well as the tumors being treated, are considered tumor-associated antigens. Antibodies that recognize tumor-associated carbohydrate antigens have frequently been observed in cancer patients. Certain anti-glycan antibodies have been shown to be involved in tumor immunosurveillance (17) and regulating autoimmunity (18), and, in some cases, anti-glycan antibodies have shown promise for early detection and prognosis of cancer [for example, (19, 20)]. Much less, however, is known about this family of antibodies as treatment specific biomarkers.

While many anti-glycan antibodies may contribute to clinical outcome, *a priori* prediction of the most relevant glycans remains difficult. Technical limitations prevent the characterization of any virus or organism's glycome, and conventional assays for immunogenic properties of individual glycans remain arduous. Glycan microarrays (21, 22), which contain hundreds of carbohydrates immobilized on microscope slides, overcome many of these technical limitations by providing a multiplex assay for profiling serum anti-glycan antibodies requiring only minute amounts of available glycans and clinical samples.

We used glycan microarrays as part of a glyco-immunomics strategy (high-throughput profiling of serum anti-carbohydrate antibodies) to evaluate whether pre-vaccination levels of serum anti-glycan antibodies correlate with clinical response to PROSTVAC-VF, a poxvirus-based therapeutic cancer vaccine in phase III clinical trials for the treatment of advanced prostate cancer. PROSTVAC-VF induces immunity for prostate specific antigen (PSA) using genetically modified vaccinia and fowlpox encoding PSA and three co-stimulatory molecules (LFA-3, B7.1, and ICAM-1) co-administered with GM-CSF. In two phase II clinical trials (23, 24), PROSTVAC-VF treatment was associated with preliminary evidence of a 8–9 months improvement in median overall survival. While PROSTVAC-VF shows promise and has entered a phase III trial (25), not all patients experience improved survival. Since PROSTVAC-VF uses viral vectors that are glycosylated, generates responses to a glycoprotein (PSA), and stimulates immunity to tumors that display tumor-associated carbohydrate antigens, we hypothesized that pre-existing anti-glycan antibody levels influence clinical outcomes.

In this study, we demonstrate that survival outcomes for PROSTVAC-VF correlate with humoral immunity for a blood group A-like glycan found on its viral vectors. These antibodies provide a promising emerging biomarker for predicting an individual patient's response to PROSTVAC-VF. Additionally, these results suggest anti-glycan antibodies are an underexplored reservoir of biomarkers for personalized medicine.

Patients and Methods

Serum samples

Sera from PROSTVAC-VF recipients (n = 28 for training; n = 76 for validation) and controls (n = 37) came from phase II clinical trials (23, 24). Sera were stored at -70°C . Clinical data were blinded during data collection and processing.

High-throughput profiling of serum anti-glycan antibodies

Serum anti-glycan antibodies (diluted 1:50 and 1:200) were profiled on a glycan microarray (204 array components listed in Supplemental Table 1), which previously has been validated with numerous monoclonal antibodies and lectins (26–28) and shown to have excellent reproducibility (29). Arrays were printed as previously described (30, 31) except SMP2 pins (TeleChem) replaced SMP3 pins, and the print buffer included DyLight 649 (0.7 $\mu\text{g/mL}$; Thermo Scientific) to assess print quality before being washed away prior to serum assays. Bound anti-glycan antibodies were detected with fluorescent secondary antibodies (Jackson ImmunoResearch) specific for IgM, IgG, or total immunoglobulin.

Because >800 arrays were required, experiments were designed to minimize technical variations and help assess quality (32). Slides came from the fewest possible print batches, and consistency of print batches was checked using reference serum. Samples were analyzed in a random order to ensure intermixing of controls and vaccinated subjects with varied responses to PROSTVAC-VF. Additionally, the same experimenter collected all array data, and samples were analyzed on replicate slides to identify technical faults.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (33) and are accessible through GEO Series accession number GSE42184 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=hzafteseseemfa&acc=GSE42184>).

Glycan profiling of viral vectors

Glycans on viral vectors were assayed by adapting the glycan microarray for a competitive binding assay. Serum (diluted 1:500 in 1% BSA and 0.3% HSA) was pre-blocked for 1.5 hours at 37° C with vaccinia (9.1E9 pfu) and fowlpox (6.9E10 pfu) that had been propagated in chicken embryo dermal cells, purified with a sucrose gradient, and UV inactivated. Presence of particular glycans on viral vectors was detected as reduced binding of pre-blocked serum relative to binding of control serum.

Second, ELISA measured BG-A glycans on viral vectors. Viral vectors (diluted to 10 µg/mL in carbonate buffer, pH = 9.6) were coated onto Maxisorp plates (Nunc) and incubated with monoclonal anti-BG-A antibody HE-195 (GeneTex; diluted 1:100 in PBS + 3% BSA). Next, bound monoclonal antibody was detected with AP-conjugated secondary antibody (Jackson ImmunoResearch; diluted 1:250) that catalyzed the conversion of methylumbelliferylphosphate (Sigma; 26 µg/mL in Tris pH = 9.0) into a fluorescent indicator.

Measurements of overall titers to viral vectors

Assays of anti-vaccinia and anti-fowlpox IgG titers were previously reported (23, 24, 34).

Statistical analyses

Training set leads were identified as statistically significant survival differences between subjects with high versus low pre-vaccination antibody levels (non-parametric log rank p -value <0.05 for Kaplan-Meier survival estimator). To avoid noise at low antibody signals, a minimum threshold (seven times the background signal) was set for stratifying subjects. Additionally, all strata needed 7 subjects since disproportionate strata (e.g., 5 versus 23 subjects) show chance survival differences more frequently than predicted by log rank p -values.

Due to the potential for overfitting of data, lead validation required consistent, statistically significant survival differences after accounting for multiple comparisons by permutation testing (34). Probability of type I error (α) was estimated as the frequency of random permutations (3,000 runs) with 1 lead showing survival differences, at thresholds determined by the training set, with equal or better significance than observed for the lead (e.g., for BG-A_{tri} IgM, difference in median survival = 14.7 months and p = 0.005).

Correlations between anti-glycan antibodies and clinical or other immunologic data were assessed by non-parametric Kendall correlations (τ) and their associated p -values calculated using Partek Genomics Suite.

Results

Pre-vaccination antibodies to blood group A predict post-vaccination survival

Serum anti-glycan antibodies of PROSTVAC-VF recipients were profiled with glycan microarrays (Fig. 1A) containing 171 structurally distinct glycans (Supplementary Table 1) (26, 29, 30). We tested for survival correlations with anti-glycan antibody levels in two stages (training and validation) in order to reduce false positives common when performing multiple comparisons. The training stage used sera from 28 subjects enrolled in a single center, phase II study of PROSTVAC-VF for metastatic castration-resistant prostate cancer

(24). Next, potential biomarkers from the initial training set were validated in an independent set of 76 patients from a separate, multi-center phase II study (23). As controls, samples were obtained from 37 prostate cancer subjects inoculated with wild-type vectors (vaccinia and fowlpox lacking the 4 transgenes) co-administered with saline injections instead of GM-CSF (23). In each case, all clinical data were blinded until after completion of antibody profiling.

Of 39 leads identified by the training set (Supplementary Table 2), only pre-vaccination levels of serum IgM antibodies to blood group A trisaccharide [BG-A_{tri} = GalNAc α 1-3(Fuc α 1-2)Gal β 1] were statistically significant in both the training ($p = 0.04$; Fig. 2A) and validation sets ($p = 0.005$; Fig. 2B) after correcting for multiple comparisons ($\alpha = 0.036$). That is, there was a 3.6% likelihood that chance would produce a survival correlation for any lead equivalent to or better than the survival correlation observed for anti-BG-A IgM. Median survival for subjects with abundant IgM for BG-A_{tri} was significantly longer than for subjects with little or no anti-BG-A_{tri} IgM. Upper quartiles for pre-vaccination anti-BG-A_{tri} IgM survived nearly twice as long as the lowest quartile [median survival of 13.9 vs. 33.7 months in training set ($p = 0.05$); 17.1 vs. 31.0 months in validation set ($p = 0.01$)]. Additionally, median actual survival for the upper quartiles was 11.5–15.7 months longer than their median Halabi predicted survival (training = 18.0 months; validation = 19.5 months) and 14.4 – 17.1 months longer than the median survival of controls (16.6 months). There was no survival correlation with anti-BG-A_{tri} IgG.

Our two-stage analysis was designed to validate potential biomarkers at the same threshold in both training and validation sets in order to guard against overfitting. The small training set, however, was not adequately powered to evaluate extreme cutoff values since estimates based on strata containing few subjects are prone to small sample size effects (35). The larger number of subjects in the validation set allowed us to more accurately evaluate cutoff values for Kaplan-Meier analysis. At the optimal cutoff, where approximately 22% of the patients were below the threshold, the survival correlation was even stronger ($p = 3E-6$; Fig. 2C). Therefore, performance of anti-BG-A_{tri} IgM as a biomarker may be improved as larger numbers of patients are evaluated and the cutoff is further refined.

Anti-BG-A_{tri} IgM are prognostic specifically for vaccinated subjects

As a first step in investigating the biological basis for improved survival in subjects with abundant serum levels of IgM for BG-A_{tri} prior to vaccination, we assessed whether this correlation was specific for vaccinated subjects or whether anti-BG-A_{tri} IgM are generally prognostic. We profiled serum anti-glycan antibodies of control subjects inoculated with wild-type viral vectors. There was no correlation with survival for subjects treated with control vectors (Fig. 2D); therefore, levels of anti-BG-A_{tri} IgM were specifically predictive of survival after treatment with PROSTVAC-VF. Also, we evaluated relationships between anti-BG-A_{tri} IgM levels and measures of disease severity, such as PSA level and Gleason score. Third, we tested for associations with prognostic factors, such as Halabi predicted survival (36) and age. No correlation with these factors was observed (Fig. 3), indicating the link between survival and anti-BG-A_{tri} IgM is unlikely to be an epiphenomenon.

Array's diversity helps refine structure of antigen associated with improved survival

High-throughput glyco-immunomic profiling provided considerable information about the binding properties of the antibody populations associated with vaccine efficacy. The blood group A determinant is defined as the trisaccharide, GalNAc α 1-3(Fuc α 1-2)Gal; however, this trisaccharide can be found in nature attached to various oligosaccharide chains. Six distinct tetrasaccharide variants of the blood group A antigen have been described (referred to as blood groups A1 through A6). In addition, the blood group H and B antigens share

considerable structural similarities with the blood group A antigen. The array contained many structurally-related glycans (Fig. 4), but survival correlated with pre-vaccination levels of IgM to BG-A_{tri} uniquely. Interestingly, all substitutions, eliminations, or truncations of BG-A_{tri} weakened the survival correlation (Table 1). The low degree of correlation between antibody binding for structurally related glycans indicated that distinct antibody populations recognize each structural variant. Improved survival, in particular, was not associated with antibodies for a common blood group A variant in humans (BG-A1 = GalNAc α 1-3(Fuc α 1-2)Gal β 1-3GlcNAc β 1-3Gal β 1), which are strongly associated with blood type (29) and previously have been found to be distinct from anti-BG-A_{tri} IgM (37). Antibodies to blood groups B and H did not correlate with survival. There was no survival correlation for the terminal monosaccharide GalNAc- α or for peptides containing the tumor associated Tn antigen (GalNAc α linked to serine or threonine). Additionally, IgG antibodies for BG-A_{tri} did not correlate with survival, although serum levels and variation between subjects were similar for IgG and IgM antibodies. Taken together, enhanced vaccine efficacy appears most closely associated with IgM antibodies for a blood group A-like glycan distinct from human BG-A1.

PROSTVAC-VF's viral vectors display blood group A-like glycan

Antibodies to BG-A_{tri} described above could potentially contribute to PROSTVAC-VF's clinical efficacy in several ways, such as by binding malignant cells and exerting a direct anti-tumor effect or by binding viral vectors and facilitating their trafficking, uptake, and presentation. While we cannot exclude mechanisms in which antibodies exert direct anti-tumor effects, the poor correlation between BG-A_{tri} levels and survival in the control patients led us to evaluate alternative mechanisms.

Viruses frequently display host cell glycans on their surface. The PROSTVAC-VF poxviruses are propagated in chicken embryo dermal cells, which are known to biosynthesize blood group A-like glycans (38). Vaccinia (39) and other vaccines (40, 41) produced in chicken cells have long been recognized to contain a blood group A-like glycan. Therefore, we hypothesized that the BG-A_{tri} antibodies detected in our array profiling may recognize glycans on the viral vectors. To test this hypothesis, we profiled glycans on wild-type poxviruses using a competitive binding assay (Fig. 5A). Purified poxviruses depleted nearly all serum IgM binding to BG-A_{tri}, its defucosylated disaccharide (BG-A_{di} = GalNAc α 1-3Gal), and the terminal disaccharide of the Forssman antigen (Fs_{di} = GalNAc α 1-3GalNAc). Reduction of antibody binding to BG-A and Forssman glycans was substantially larger than the non-specific adsorption of other antibodies to the poxviruses. Notably, the glycan profile of purified poxviruses is consistent with the glycosylation of chicken embryos, which produce both blood group A-like glycans and Forssman antigen (42). ELISA confirmed the presence of BG-A_{tri} on the viral vectors (Fig. 5B). Furthermore, we have previously observed antibody responses to BG-A_{tri} in patients vaccinated with PROSTVAC-VF, providing additional evidence of BG-A-like glycans on the poxvirus surface (26). Interestingly, although PROSTVAC-VF's vectors apparently contain BG-A_{tri}, levels of antibodies to these glycans were unrelated to pre-vaccination overall anti-vaccinia IgG titers (Fig. 6). This indicates that anti-glycan immunity provides distinct information from overall titers, which are poor predictors of post-vaccination survival (23).

Discussion

Cancer vaccines can produce durable responses in some patients, but others receive little or no immediately observable benefit. Critical determinants of *in vivo* vaccine efficacy are poorly understood. In this study, we demonstrate that pre-existing serum antibody levels to BG-A_{tri} correlate positively with overall survival for patients treated with PROSTVAC-VF. The correlation was statistically significant in patients from two separate phase II clinical

trials encompassing over 100 patients and remained statistically significant even after correcting for multiple comparisons. In addition, the survival correlation was specific to PROSTVAC-VF since antibody levels were: [1] independent of disease severity (PSA levels, Gleason score), [2] independent of other prognostic factors (Halabi predicted survival and age), and [3] not correlated with survival in control patients inoculated with wild-type viral vectors lacking PSA and co-stimulatory transgenes.

The survival correlation of anti-BG-A_{tri} IgM in vaccinated subjects is intriguing. BG-A_{tri} is the terminal trisaccharide portion of the blood group A antigen, which is expressed at high levels on red blood cells, epithelia, epidermis, and mucins of blood type A and AB individuals (43). Although the biological roles of ABO blood group antigens are still not well understood, it is known that antibodies to non-self ABO blood group antigens develop early on in life and are maintained at relatively constant levels in healthy adults (29, 44). These antibodies have been proposed to provide a barrier to curb the spread of certain diseases across individuals of different blood types (45). Moreover, strong immune responses to “foreign” ABO blood group antigens limit incompatible blood transfusions and organ transplants.

In the case of PROSTVAC-VF, the anti-BG-A_{tri} IgM bind a blood group A-like glycan on the surface of the poxviruses. The glycan is presumably carried over from chicken embryo dermal cells, the host cells used for production of PROSTVAC-VF. Antibody bound to viral vectors can influence immune responses in a variety of ways. For example, emerging evidence indicates that certain types of pre-existing antibodies, referred to as “natural antibodies” or B-1 class antibodies, may augment vaccine responses by linking innate and adaptive immunity (46). These natural antibodies, which include many anti-glycan antibodies, have been shown in murine models to enhance immune responses in a complement-dependent manner (47). Studies on the α -gal antigen (48, 49) provide additional precedent for carbohydrates as vaccine adjuvants. Binding of pre-existing antibodies to the α -gal glycan on vaccines has been shown to enhance antigen uptake and presentation and induce stronger immune responses (50, 51). In fact, attachment of the α -gal glycan to antigens can significantly enhance their immunogenicity (50, 51). Anti-BG-A_{tri} IgM binding to PROSTVAC-VF’s viral vectors may increase immunogenicity in a similar manner.

Although other pre-existing serum antibodies may bind the poxvirus vectors, anti-BG-A_{tri} IgM appear to be unique. Pre-existing antibodies for a vaccine’s viral vectors have long been investigated as potentially influencing vaccine efficacy. For example, several studies have evaluated relationships between overall antibody titers to poxvirus vectors and survival (23, 24). These studies have primarily grown out of concerns that pre-existing neutralizing antibodies to the vectors would render virus-based vaccines ineffective; however, these studies showed no correlation between overall titers to poxvirus vectors and survival. Moreover, there are numerous other glycans on the poxvirus surface; of the anti-glycan antibody populations detectable on our array, however, only pre-existing anti-BG-A_{tri} IgM correlated with clinical outcome, possibly due to a higher level of expression of the corresponding antigen on the viral surface than other glycans, a unique location of the antigen, or more substantial variability in anti-BG-A_{tri} IgM levels among patients. It is important to note that antibody-mediated immune enhancement could occur in patients receiving either PROSTVAC-VF or the wild-type control poxviruses; however, the correlation of anti-BG-A_{tri} IgM with *survival* was only observed in patients treated with PROSTVAC-VF. Therefore, these antibodies only correlate with clinical outcome when coupled with immune stimulation towards a tumor antigen.

Our results have a number of important implications. They provide the first evidence that anti-carbohydrate antibodies could be useful biomarkers for PROSTVAC-VF. In principle, potential patients may be pre-screened for IgM antibodies to BG-A_{tri} as one criterion for treatment with PROSTVAC-VF. Although our study comprised over 100 vaccinated patients, as well as controls to demonstrate the prognostic specificity of anti-BG-A_{tri} IgM, additional prospective studies with larger numbers of patients and control subjects will be an important focus of future research. The ongoing Phase III clinical trial on PROSTVAC-VF is anticipated to enroll 1,200 patients, and samples from this study could provide additional validation of anti-BG-A_{tri} IgM as a predictor of clinical response (25). Second, glycan composition and consistency may be critical features of vaccine potency. Factors that influence glycan expression, such as type of host cell and growth conditions, may significantly affect clinical outcomes for pox-virus based vaccines, and optimizing these factors may improve efficacy. Analysis of a viral vector's glycans also may be an important quality control assessment. Third, anti-BG-A_{tri} IgM may be relevant to other pox-based therapies, such as other cancer vaccines [e.g., PANVAC (52) and rV-NY-ESO-1 (53)], oncolytic poxviruses (54, 55), and HIV vaccines [e.g., ALVAC-HIV (56)]. Since these poxviruses are often produced in chicken-derived cells, they also could display blood group A-like glycans on their surfaces. Therefore, antibodies to BG-A_{tri} could influence their clinical efficacy too. Analogously, antibodies levels to blood group B may be relevant for vaccines propagated in other cell lines that express blood group B. By addressing technical challenges in studying carbohydrate-protein interactions, glycan microarrays provide a powerful tool for exploring connections between anti-glycan antibodies and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Translational Relevance

Cancer vaccines can be very effective for treating patients, but clinical responses vary widely from one patient to another. Strategies to target treatment to likely responders could significantly improve clinical efficacy, minimize unnecessary side effects, and reduce health care costs. In this study, we demonstrate that overall post-vaccination survival for patients treated with PROSTVAC-VF correlates with pre-existing serum IgM levels for the blood group A glycan. Anti-blood group A levels did not correlate with survival in control patients and were independent of various measures of disease severity. Therefore, measurement of serum anti-blood group A levels prior to vaccination could provide a simple method for pre-selecting patients that are likely to benefit from PROSTVAC-VF therapy. Finally, these studies emphasize the potential of serum anti-glycan antibodies as a convenient, understudied reservoir of biomarkers to advance personalized medicine and suggest new avenues for improving poxvirus based vaccines.

Fig. 1.

Overview of glyco-immunomics. A comprehensive profile of an individual's serum anti-glycan antibodies was obtained using 5 µL of serum and a glycan microarray containing 220 features robotically spotted onto a glass slide. Analogous to protein antigen arrays, captured antibodies were labeled with fluorescent secondary antibodies, and scanning quantified the amount of IgM, IgG, or total immunoglobulin bound to each glycan.

Fig. 2. Kaplan-Meier survival curves for subjects stratified by levels of pre-existing anti-BG-A_{tri} IgM. (A) training set. (B) validation set. (C) validation set using a lower threshold. (D) control subjects. The same threshold was applied for stratifications in panels A, B, and D.

Fig. 3.

Correlation of clinical data with anti-BG-A_{tri} IgM. Sera collected prior to vaccination with PROSTVAC-VF were analyzed on the glycan microarray to determine levels of IgM antibody for BG-A_{tri}. Pre-vaccination serum levels of anti-BG-A_{tri} were plotted against clinical data for all 141 subjects. Glycan microarray data were reported as fluorescence units that have been normalized to allow comparisons across multiple array experiments. The Halabi predicted survival was calculated from a nomogram that considers 7 prognostic factors: PSA, Gleason score, serum lactate dehydrogenase (LDH), alkaline phosphatase, hematocrit, presence of visceral disease, and ECOG functional status. Correlations were assessed with Kendall correlation coefficients (τ) and their associated p -values.

Fig. 4.
Microarray components with structural similarities to BG-A_{tri}.

Fig. 5. Glycan profiling of PROSTVAC-VF's viral vectors. (A) In a blocking assay, serum was pre-incubated with PROSTVAC-VF's viral vectors prior to profiling serum antibodies on the array. IgM binding of blocked samples was normalized to IgM binding under standard conditions. Pre-incubation with PROSTVAC-VF's viral vectors markedly decreased IgM binding to BG-A glycans and Forssman disaccharide, whereas IgM binding to other glycans were reduced non-specifically. [Error bars indicate standard deviation ($n = 4$). Significance by One-way ANOVA $p = 2E-17$ for vaccinia; $p = 2E-16$ for fowlpox] (B) ELISA for blood group A on vaccinia and fowlpox. Black bars represent the mean signal from samples labeled with a monoclonal antibody specific for blood group A. Grey bars are mean

background signals from samples not incubated with a primary antibody. [Error bars indicate SEM (n = 3). * indicates $p < 0.01$]

Fig. 6.

Comparison of pre-vaccination anti-BG-A IgM and overall anti-vector IgG titers. Subjects had widely variable levels of anti-BG-A_{tri} IgM prior to vaccination. Also, many subjects had detectable levels of IgG for vaccinia prior to inoculation with PROSTVAC-VF, presumably due to earlier vaccination for smallpox. All subjects had undetectable levels of anti-fowlpox IgG prior to vaccination.

Table 1

Structural specificity of antigen associated with improved overall survival. For each glycan structurally similar to BG-A_{tri}, the table contains the minimum log-rank p -value of all possible Kaplan-Meier survival curves stratified by IgM antibody levels for that particular glycan. Also listed are the numbers of stratifications (65 possible containing 6–70 subjects) whose Kaplan-Meier survival curves have log-rank p -values <0.005. The degree of cross-reactivity of anti-BG-A_{tri} IgM for structurally similar glycans was estimated by Kendall correlations (τ) between IgM antibodies levels for BG-A_{tri} and related glycans.

	Minimum p -value	Stratifications with $p < 0.005$	Correlation (τ) with BG-A _{tri}
BG-A _{tri}	1.39E-07	26	1
BG-A _{di} (4/BSA)	1.96E-05	5	0.59
GalNAc α 1-6Gal β (22/BSA)	5.02E-04	2	0.29
GalNAc- α (4/BSA)	0.0015	0	0.29
BG-A _{di} (17/BSA)	0.0017	0	0.58
Ac-A-Tn(Thr)-S-G (5/BSA)	0.0019	0	0.26
GalNAc- α (22/BSA)	0.0031	0	0.25
GalNAc α 1-6Gal β (4/BSA)	0.0061	0	0.26
Ac-A-Tn(Thr)-S-G (23/BSA)	0.0067	0	0.34
Ac-A-Tn(Thr)-S-G (8/BSA)	0.0073	0	0.26
BG-A1	0.0083	0	0.05
BG-H2	0.014	0	0.26
Fs _{di} (31/BSA)	0.019	0	0.43
BG-B _{tri}	0.029	0	0.35
Fs _{di} (21/BSA)	0.048	0	0.43
BG-A + Lewis B hexa	0.063	0	0.62
Fs _{di} (4/BSA)	0.066	0	0.43
BG-H1	0.094	0	-0.07
BG-B _{di}	0.15	0	0.29