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Exploring binding and effector functions of natural human antibodies using synthetic immunomodulators

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Abstract

The ability to profile the prevalence and functional activity of endogenous antibodies is of vast clinical and diagnostic importance. Serum antibodies are an important class of biomarkers and are also crucial elements of immune responses elicited by natural disease causing-agents as well as vaccines. In particular, materials for manipulating and/or enhancing immune responses toward disease-causing cells or viruses have exhibited significant promise for therapeutic applications. Antibody-recruiting molecules (ARMs) – bifunctional organic molecules that re-direct endogenous antibodies to pathological targets, thereby increasing their recognition and clearance by the immune system – have proven particularly interesting. Notably, although ARMs capable of hijacking antibodies against oligosaccharides and electron-poor aromatics have proven efficacious, systematic comparisons of the prevalence and effectiveness of natural anti-hapten antibody populations have not appeared in the literature.

Herein we report head-to-head comparisons of three chemically-simple antigens, which are known ligands for endogenous antibodies. Thus, we have chemically synthesized bifunctional molecules containing 2,4-dinitrophenyl (DNP), phosphorylcholine (PC) and rhamnose. We then used a combination of ELISA, flow cytometry, and cell-viability assays to compare these antigens in terms of their abilities both to recruit natural antibody from human serum and also to direct serum-dependent cytotoxicity against target cells. These studies have revealed rhamnose to be the most efficacious of the synthetic antigens examined. Furthermore, analysis of 122 individual serum samples has afforded comprehensive insights into population-wide prevalence and isotype distributions of distinct anti-hapten antibody populations. In addition to providing a general platform for comparing and studying anti-hapten antibodies, these studies serve as a useful starting

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Supporting Information
Detailed experimental procedures and compound characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

point for the optimization of antibody-recruiting molecules and other synthetic strategies for modulating human immunity.

Introduction

Synthetic immunology, or the development of synthetic systems capable of modulating immune responses, has emerged as an exciting area of research in recent years.(1) Activities in this field have given rise to several elegant therapeutic strategies, including the development of bifunctional molecules capable of hijacking antibodies to disease-causing cells or virus particles. These synthetic compounds – termed antibody-recruiting molecules, or ARMs – simultaneously bind disease-relevant target cells and antibody proteins, and have been shown to initiate antibody-mediated immune responses, including complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and antibody-dependent cellular phagocytosis (ADCP).(2)

Although certain ARM-based strategies have required pre-immunization or addition of purified antibody proteins to achieve immune responses, approaches that leverage antibodies already present in the human bloodstream have proven particularly promising. Such strategies have the advantage of exploiting pre-existing components of healthy human serum as the mediators of cytotoxicity. Several pre-existing antibody populations have been explored for use in these approaches, including those recognizing the α-gal trisaccharide(3, 4) and 2,4-dinitroaniline (DNP).(5–10) Therefore, a comprehensive understanding of the relative abundances and cytotoxic capabilities of endogenous antibody populations would be highly enabling to ARM-based strategies. Indeed, several ELISA- and microarray-based methods have been used to detect and compare relative levels of serum antibodies, and particularly, significant interest has been focused on developing platforms to evaluate carbohydrate-antibody interactions.(11, 12) Despite a clear need, studies that directly compare the quantity and/or functional capacity of these antibody populations have not been disclosed.

Herein we compare three low molecular weight antigens for their ability to bind antibodies in normal human serum, and template cytotoxicity of antigen-labeled target cells. The three antigens we examine – 2,4-dinitrophenyl (DNP), phosphorylcholine (PC), and α1-S-rhamnose (rhamnose) – are simple, synthetically tractable materials, whose precursors can be easily obtained and incorporated into bifunctional molecules in less than five synthetic transformations. Thus, we have designed and synthesized antigen-containing constructs capable of covalently attaching to cell surfaces, binding antibodies from human serum, and directing immune effector responses against target cells (Figure 1). This platform has enabled us to compare serum samples from individuals who span a range of ages, ethnicities, and geographic locales. Our data suggests that each of these three antigens is widely-distributed throughout the human population, capable of recruiting antibody from healthy human serum, and giving rise to highly specific cytotoxic effects. Notably, of the three antigens tested, rhamnose has proven most efficacious in templating complement-dependent cytotoxicity. Furthermore, population-wide prevalence trends have also afforded insights into the origins of anti-hapten antibody populations in human serum. Thus, in addition to providing useful insights into the optimization of ARM-based therapies, data reported herein will help enable broad-based development of synthetic systems for modulating human immune responses.

Results and Discussion

To evaluate the relative antigen-binding and cytotoxic properties of different natural antibodies, we designed and synthesized N-hydroxysuccinimide (NHS) esters **1–5** (Figure 2,

see Supporting Information for synthetic details). NHS esters are known to form covalent bonds with amines in the form of amide linkages, and are routinely used as reagents to label free N-termini and lysine residues of proteins.(13) We therefore envisioned that constructs **1–5** could be used to covalently attach similar quantities of the candidate antigens to the extracellular surfaces of cells. Molecule **4**, which contains a benzyl carbamate (Cbz) instead of a candidate antigen, was designed to serve as a negative control for use in these studies. Notably, pilot experiments have demonstrated that the relative reactivity of NHS esters **1–5** are not significantly perturbed by the incorporated antigen (Supporting Information Table S1 and Figure S1)

To compare the relative amount of each anti-hapten antibody population in human serum, we first functionalized A172 glioblastoma cells with NHS esters **1–5**. Hapten-derivatized cells were then incubated with human serum, treated with several isotype-specific phycoerythrin-labeled secondary antibodies, and relative fluorescence values were quantified using flow cytometry. Notably, we chose A172 cells in these experiments because of their documented expression of complement inhibitors,(14) thus preventing complement-dependent lysis of functionalized cells.

DNP, PC, and rhamnose antigens were all found to bind a significant amount of antibody from human serum compared to background, yet the relative quantities of IgG and IgM isotypes differed substantially between haptens (Figure 3A, B). In particular, PC was found to bind substantially more IgM antibody than the other motifs – a finding consistent with previous reports(15) – while rhamnose was found to bind significantly more IgG antibody. To confirm antibody-antigen specificity, we conducted serum-binding experiments, which indicated that antibody binding could be competitively inhibited by rhamnose in a concentration-dependent fashion. A negligible amount of antibody binding was observed under control conditions involving the Cbz antigen, further suggesting that antibody binding resulted from specific interactions with antigens, and not linker motifs or neo-epitopes formed from covalent modification of cell surfaces.

We next compared the relative amounts of each IgG antibody subclass – IgG1 through IgG4 – bound by the three antigenic motifs.(8, 16–19) Human IgG1 is the most abundant subclass of IgG, comprising approximately 70% of total IgG.(16) Furthermore, IgG1 has been shown to be potently cytotoxic compared to other IgG sub-isotypes, perhaps because of its strong interactions with Fc RIIIa on natural killer cells,(17) and the complement protein C1q.(17, 18) Indeed, perhaps for this reason, seven of the eight non-conjugated monoclonal antibodies approved by the FDA for treating cancers are from the IgG1 subclass.(17, 20) IgG2, on the other hand constitutes only approximately 20% of total IgG.(16) Although it does not activate cytotoxic or phagocytic pathways, it is believed to serve an important role in suppressing bacterial infections.(16, 21) Human IgG3 comprises approximately 7% of total IgG,(16) and like IgG1, is effective at activating antibody-dependent CDC, ADCC, and ADCP pathways.(19) IgG4 is the scarcest subpopulation (comprising approximately 3% of total IgG)(16) and is the least effective subtype for initiating immune responses.(19)

Synthetic antigens **1–3** all bind IgG antibody with a substantially different subclass profile (Figure 3D). The rhamnose antigen, which binds the most total IgG, interacts predominantly with IgG1 and IgG2 subclasses. This observation is consistent with previous reports showing that immunization with sugar-derived antigens often leads to predominantly IgG2 responses.(22) The PC antigen, on the other hand, mostly binds IgG2 and IgG3 isotypes, while the DNP antigen binds antibody mostly of the IgG1 isotype. Taken together, these data suggest that the three antigens tested likely induce antibody production through different mechanisms, and all of them are likely capable of initiating both cytotoxic and phagocytic immune responses *in vivo*.

We next used the synthetic NHS-ester-labeling platform to study ligand-dependent cytotoxicity. For these experiments we employed a cell-viability assay based on the commercially-available CellTitreGlo reagent, which has been well-validated for use in measuring CDC (see supporting information for details).(10) Chinese hamster ovarian cells (CHO) were used in these experiments because, unlike A172 cells, they do not express cell-surface complement inhibitors, and should therefore be susceptible to the cytotoxic effects of antibody and complement proteins found in human serum. Indeed, significant increases in serum-dependent cytotoxicity were observed in cells derivatized with antigens **1–3** versus unmodified cells or cells functionalized with Cbz antigen **4**, which does not recruit significant endogenous antibodies (Figure 4). Among synthetic antigens **1–3**, rhamnose led to the highest levels of cell death, while DNP and PC antigens were found to be less active. While IgM, IgG1, and IgG3 antibody are all known to be capable of initiating the complement cascade by localizing C1q to cell surfaces,(18, 19) the levels of cytotoxicity observed in these experiments roughly correlate with the relative total amount of IgG1 and IgG3 bound by each antigen. The relative lack of PC-induced cytotoxicity in these assays may be related to PC's ability to bind C-reactive protein (CRP), which is known to suppress the later stages of the complement cascade and inflammatory responses.(23, 24)

Improvements in the antibody-recruiting and cytotoxic abilities of mono- versus bis-functionalized antigen structures also proved noteworthy. The bis(rhamnose) antigen **5** was observed to recruit significantly more IgM and IgG antibody from the pooled serum versus the monovalent rhamnose antigen (Figure 5A, B). Furthermore, the bis(rhamnose) antigen was found to give rise to approximately twice the amount of CDC compared to the monovalent rhamnose antigen (Figure 5C). Qualitatively, these functional differences between mono- and bis-rhamnose antigens correlate with levels of antibody recruited to cell surfaces, and may be the result of multivalency effects leading to a non-linear relationship between levels of antibody opsonization and immune-mediated cytotoxicity.(3) For example, Friedman and coworkers have shown that dimeric, trimeric, and tetrameric PC molecular constructs bind to a monoclonal anti-PC IgM antibody with $>10^3$-fold increased affinity versus the monovalent antigen.(25) Additionally, Wang and coworkers have shown that a polymeric α-Gal structure binds to anti-Gal IgM antibody from serum with 10^4-fold increased affinity versus the monovalent antigen.(26) Because multivalent antigens also have the potential to aggregate antibody, thus non-selectively initiating some immune responses even in the absence of a cellular target,(27) it is not clear if multivalent or monovalent systems will be most effective and selective for directing immune responses in live-animal studies.

We next set out to measure how the quantities and innate cytotoxic capabilities of different antibody populations vary between individuals. As a sample set, we studied serum samples obtained from 122 healthy individuals who span diverse ethnic backgrounds, live in seven geographically disparate sites in the continental United States, and range from two to 88 years of age (see Supporting Information for details). To compare these samples, we first utilized an ELISA format in which 96-well plates were coated with antigen-labeled bovine serum albumin, incubated with human serum, and quantified using HRP-conjugated anti-human secondary antibody. As shown in Figure 6, the IgG and IgM populations of anti-DNP, anti-rhamnose, and anti-PC antibody show qualitatively different distributions within the sample population. The distributions of anti-DNP IgG and anti-rhamnose IgG fit well to log-normal (Galton) distributions with most values clustering near the lower extreme and outliers trailing into the upper extreme (Figure 6A, C).(28) The distributions of the three IgM populations, on the other hand, adopt values nearer to those of pooled serum, with fewer extremely low values, as compared to IgG (Figures 6B, D, F). The levels of anti-PC IgG only slightly favors the lower values (Figure 6E) and therefore appears to distribute somewhere between Galton and Gaussian patterns. These observations are consistent with a

model in which anti-PC IgG antibody are at least partially maintained by B1 cells and therefore do not fluctuate as dramatically as the B2-controlled IgG populations. Indeed, B1 cells are also know to contribute to basal levels of some IgG populations,(29, 30) and to contribute to production of antibody against self antigens including PC.(29, 31) Although B1 cells do amplify specific antibody populations in response to stimulation by matching antigens, these amplifications return to basal levels more quickly than do B2-based amplifications,(32, 33) which may explain why the anti-DNP and anti-rhamnose IgM populations are perturbed from normal Gaussian distributions, yet not as dramatically as the IgG populations.

Comparisons between IgG and IgM antibody levels within individuals have revealed an interesting trend. While there appear to be no associations between levels of anti-hapten antibodies within the IgG isotype (Figure 7A), there are strong correlations between the amount of each IgM population recruited from a given serum sample (Figures 7B, C, D, Spearman values: $0.387 \leq r \leq 0.634$, $p < 0.0001$). Taken together, these trends are consistent with the accepted model in which B1 cells help maintain basal levels of a broad range of IgM antibodies,(27, 29, 31, 32, 34) and some individuals have higher overall antibody levels, across many distinct antigens. IgG antibody levels, on the other hand, are maintained by antigen-specific B2 cells, which respond exclusively to exposure to matching antigenic compounds.(19) Therefore, while most IgG levels vary greatly due to external factors, IgM and anti-PC IgG levels remain more consistent over time and between individuals.

We also performed covariance analyses of total IgM and IgG levels in human serum, with respect to factors such as age, gender, ethnicity, and geographic location. In general, these analyses suggest the lack of any strong associations between these factors and anti-hapten antibody levels, although several weak correlations were observed. For example, small, yet statistically significant decreases in the levels of anti-rhamnose ($p = <0.0001$) and anti-PC ($p = 0.003$) IgM were observed with increasing age; however, neither anti-DNP IgM, nor any of the antigen-specific IgG populations, seemed to exhibit any significant dependence on age. This trend is supported by previous literature analyses, which have noted age-related declines in IgM levels and relatively fixed IgG levels in adults.(20) Other minor associations observed (see Supporting Information) are likely spurious, and more information will be required to conclude whether these findings reflect population-wide trends.

Finally, we compared the levels of antibody recruited from pooled and individual serum samples using flow cytometry assays. In general, these experiments revealed that levels of both IgM and IgG antibody for each antigen correlate well between pooled and individual serum samples (Figure 8A, B). Specifically, IgM levels appear to be greatest for anti-PC antibodies, followed by anti-rhamnose and anti-DNP, which are present in approximately equivalent amounts. For IgG, on the other hand, anti-rhamnose antibodies are present in the greatest quantities, followed by anti-PC and anti-DNP. Interestingly, CDC results (Figure 4) strongly correlate with trends in IgG levels, suggesting that this isotype plays a predominant role in mediating cytotoxicity. An important caveat in this analysis is the known instability of complement proteins, which likely causes substantial non-uniformity in the amount of active complement protein in each sample. Taken together, these comparisons suggest that data obtained using pooled human serum likely reflects population-wide trends, rather than the influence of a relatively small number of outlier values.

Conclusions

Although previous authors have used synthetic antigens to measure the prevalence of antibodies in human serum directed against carbohydrate antigens,(11, 12) our studies are the first to characterize the isotype distributions and compare “head-to-head” the functional

effector properties of several diverse anti-hapten antibody classes. Using synthetic constructs that contain specific antigenic motifs, combined with biochemical and cellular assay systems, our studies have demonstrated several interesting trends, which both clarify, and expand upon, previous literature. For example, we have demonstrated that there is significant variability between levels of antigen-specific IgM and IgG, and that these differences likely reflect the origins of each of these antibody populations. Whereas IgG levels arise from adaptive immune responses following antigenic exposures, the IgM antibody repertoire is maintained innately, at levels that do not reflect external factors. Indeed, IgM antibodies have been shown to be polyspecific, and capable of binding multiple antigens with relatively low affinities. The majority of IgG antibodies, on the other hand, are produced following affinity maturation, which requires the presence of a specific antigen. For example, rhamnose is a monosaccharide found in capsular structures in various pathogenic and non-pathogenic bacteria.(11) It is likely, therefore, that anti-rhamnose antibodies are produced as part of a host-defense response, and reflects the relatively high rate of exposure to these organisms. PC, on the other hand, is found in cell membranes in humans. It is believed that during apoptosis, PC antigens become exposed on the extracellular surfaces of the dying cell's membrane, and then anti-PC antibodies recognize these antigens and mediate complement-based immune responses against the target.(36–38) The IgM predominance of anti-PC antibodies is consistent with the fact that PC is a component of "self" structures. Finally, although the existence of anti-DNP antibody populations in human serum has been well documented,(35, 39) their mechanistic origin is unknown, and has been speculated to result from both environmental and innate pathways. It is not surprising in this light that the relative cytotoxic potential of anti-hapten antibodies tracks with the type of antigenic exposure that leads to their production.

In recent years there has been much interest in developing synthetic molecules capable of harnessing the immune system to target and destroy diseased cells. Several groups have reported ARM-based strategies designed to function as anti-viral,(4, 10, 21, 40) anti-bacterial,(41, 42) or anti-cancer agents.(3, 5, 8, 23, 43) Among their many advantages in comparison with traditional therapeutics, ARMs possess the unique ability to convert healthy human serum into a targeted cytotoxic agent.(13) The data reported herein expand the palette of simple chemical structures that can be exploited in ARM strategies, and have the potential to help advance areas of biomedicine ranging from disease prognosis to cellular immunotherapy and synthetic vaccine development.(44)

Methods

Detailed descriptions of materials, commercial sources for biological reagents, and experimental procedures can be found in the Supporting Information.

Materials and chemical synthesis

Organic chemicals were purchased from Sigma–Aldrich or Advanced ChemTech. Antibody reagents were purchased from Invitrogen, Bethyl Laboratory, or Southern Biotech. Functionalized BSA was purchased from Biosearch Technologies or Dextra Laboratories. Human serum was purchased from Innovative Research, Bioreclamation, or Nova Biologicals. All experiments using pooled serum were performed with the same batch, which was obtained from Innovative Research and was pooled from approximately 10–15 donors. A172 and CHO-K1 cells were purchased from ATCC, grown according to the supplier's instructions, and used within six months of resuscitation. Molecules **1–5** were synthesized using standard organic chemistry procedures and characterized by standard techniques including ^{1}H and ^{13}C NMR spectroscopy, infrared spectroscopy, and mass

spectrometry. Molecules **1–5** were purified to analytical purity by preparative reverse phase HPLC.

Flow Cytometry

A172 cells were treated with NHS-esters **1–5** or a vehicle-only control, treated with 10% v/v human serum or 10% v/v human serum with added rhamnose, and treated with phycoerythrin-labeled anti-human antibody specific for either IgM, IgG, IgG1, IgG2, IgG3, or IgG4. Cells were analyzed in the phycoerythrin channel (FL-2) by flow cytometry on an Accuri C6 flow cytometer.

Complement-Dependent Cytotoxicity Assay

CHO cells were treated with NHS-ester **1–5** or a vehicle-only control. Into the wells of a 96-well polystyrene plate were added 250,000 labeled cells and 12.5% or 0% v/v human serum. The max-kill values were obtained by adding 5% v/v hydrogen peroxide. After 3 h, the amount of live cells in each well was measured by luminescence using the CellTitreGlo viability assay using a Synergy 2 Multimode Microplate Reader.

$$\%\ CDC = \left(1 - \frac{sample - \max kill}{no\ serum - \max kill}\right) \times 100\%$$

$$(\%\ CDC\ over\ background) = (\%\ CDC\ with\ antigen) - (\%\ CDC\ of\ vehicle\ only)$$

ELISA

The wells of a 96-well polystyrene plate were coated with either BSA, DNP-labeled BSA, rhamnose-labeled BSA, or PC-labeled BSA. The wells were treated with 2% v/v human serum, followed by addition of anti-human antibody specific for IgM or IgG conjugated to horseradish peroxidase. After developing with the TMB substrate solution and quenching with sulfuric acid, the absorbance of each well was measured at 450 nm using a Synergy 2 Multimode Microplate Reader. The normalized response equals the triplicate mean divided by the triplicate mean of the pooled serum on same plate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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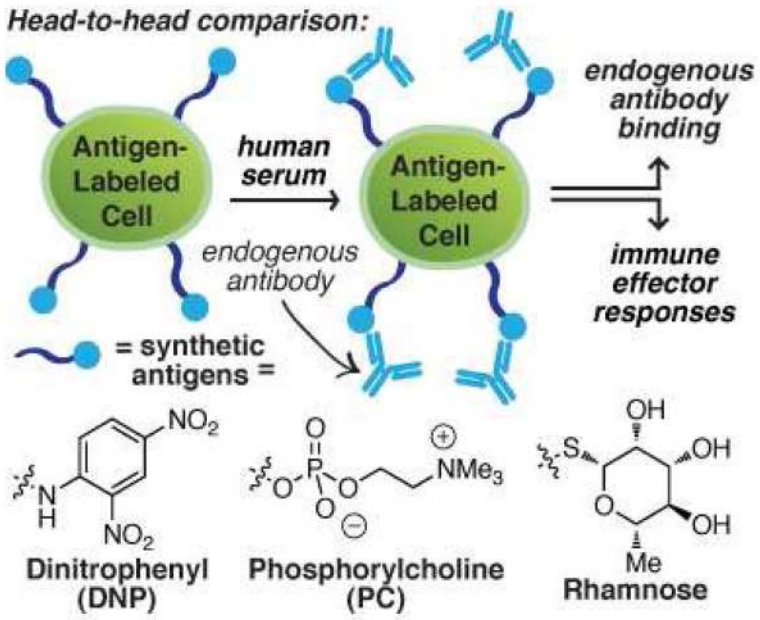

Figure 1.
Summary of the strategy used to study the ability of different chemical antigens to recruit antibody from normal human serum and to direct immune responses against labeled target cells.

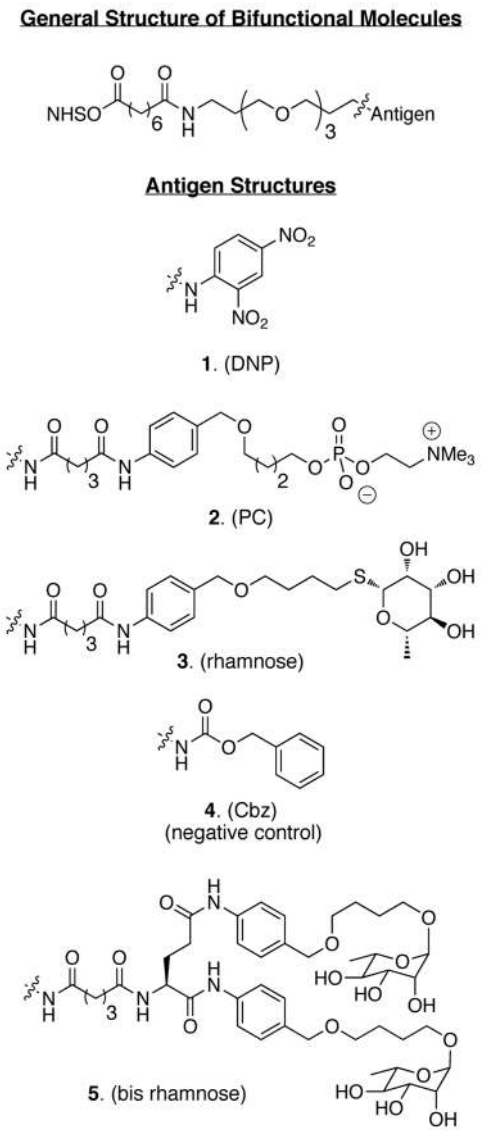

Figure 2.
Chemical structures of bifunctional molecules **1–5**, which are designed to covalently bind to cell surfaces and label the cells with specific antigenic chemical structures.

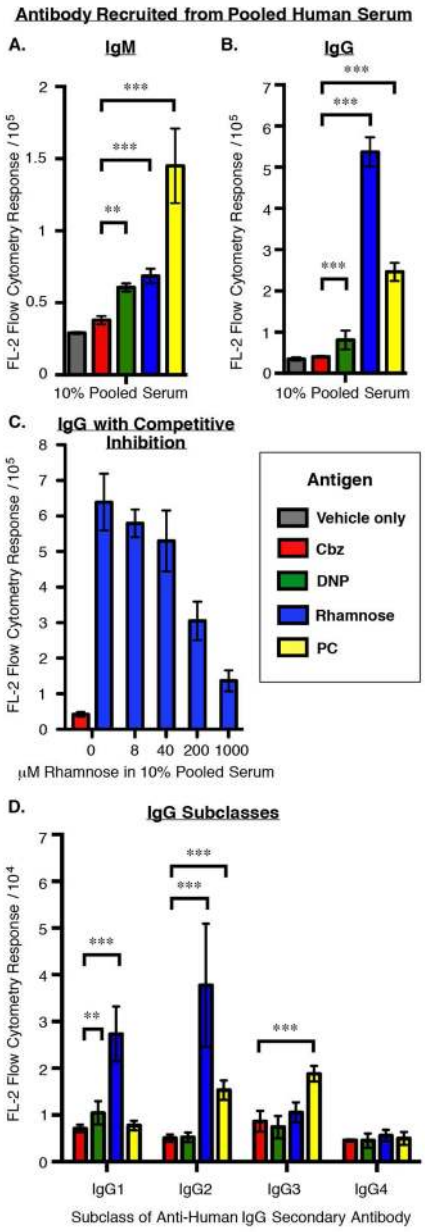

Figure 3.

Binding of antibody from human serum to cells derivatized with synthetic antigens as quantified by flow cytometry (phycoerythrin channel, FL-2). (A) IgM bound. (B) IgG bound. (C) Competitive inhibition of IgG binding by soluble rhamnose. (D) IgG subtypes bound. Data points represent mean values of 3–8 experiments. Error bars represent 95% confidence intervals. T-test comparisons at 95% confidence were performed using GraphPad Prism 5.0 (** $p < 0.01$, *** $p < 0.001$).

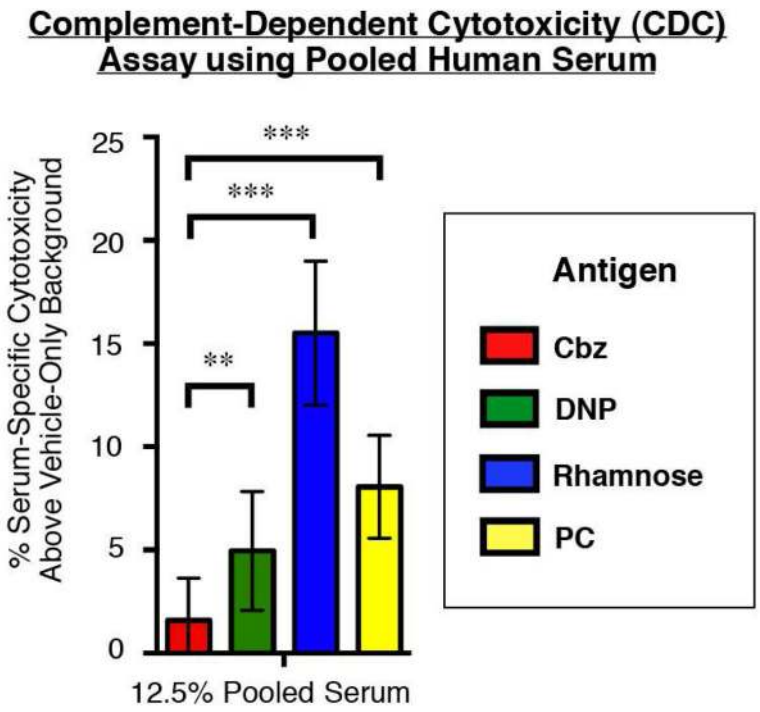

Figure 4.

Comparison of ligand-dependent cytotoxicity following labeling of CHO cells with synthetic antigens. Data points represent the mean values of 12 experiments. Error bars represent 95% confidence intervals. T-test comparisons at 95% confidence were performed using GraphPad Prism 5.0 (** $p < 0.01$, *** $p < 0.001$).

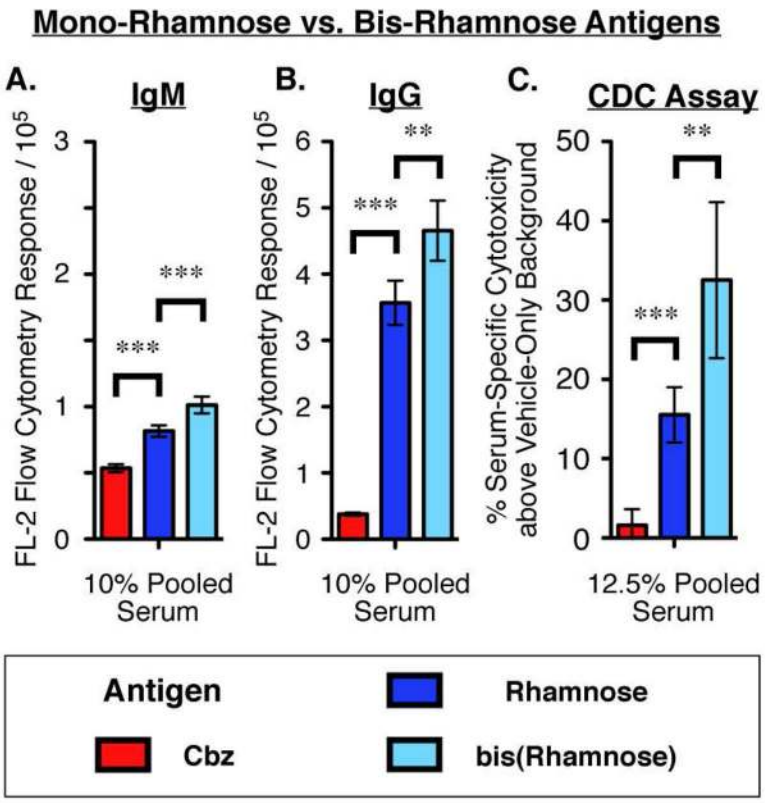

Figure 5.

Comparison of monovalent rhamnose antigen **3** to bivalent rhamnose antigen **5**. (A) IgM recruited. (B) IgG recruited. (C) CDC assay. Data points represent mean values of 3 experiments. Error bars represent 95% confidence intervals. T-test comparisons at 95% confidence were performed using GraphPad Prism 5.0 (** $p < 0.01$, *** $p < 0.001$).

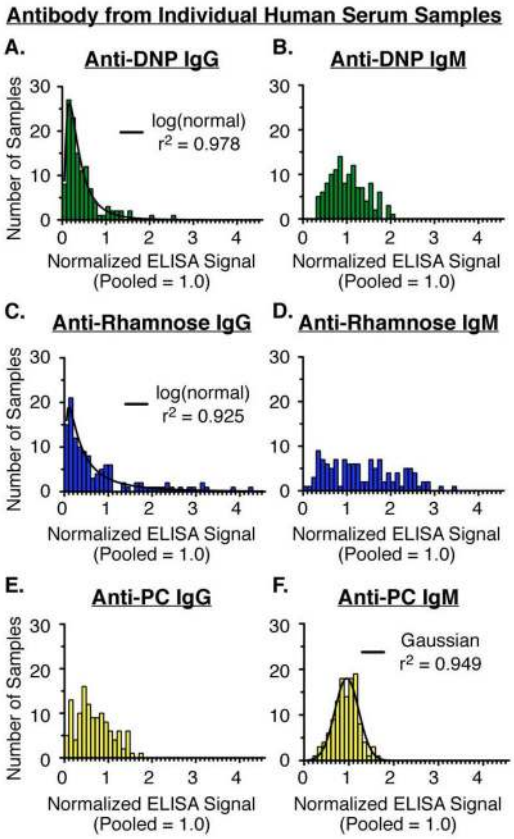

Figure 6.

Results of ELISA experiments measuring natural anti-hapten antibody levels in individual serum samples. Reported values are normalized with respect to pooled serum and represent the mean of triplicate experiments. Lines of best fit were create with the log(Gaussian) or Gaussian non-linear fitting function in the GraphPrism 5.0 software package.

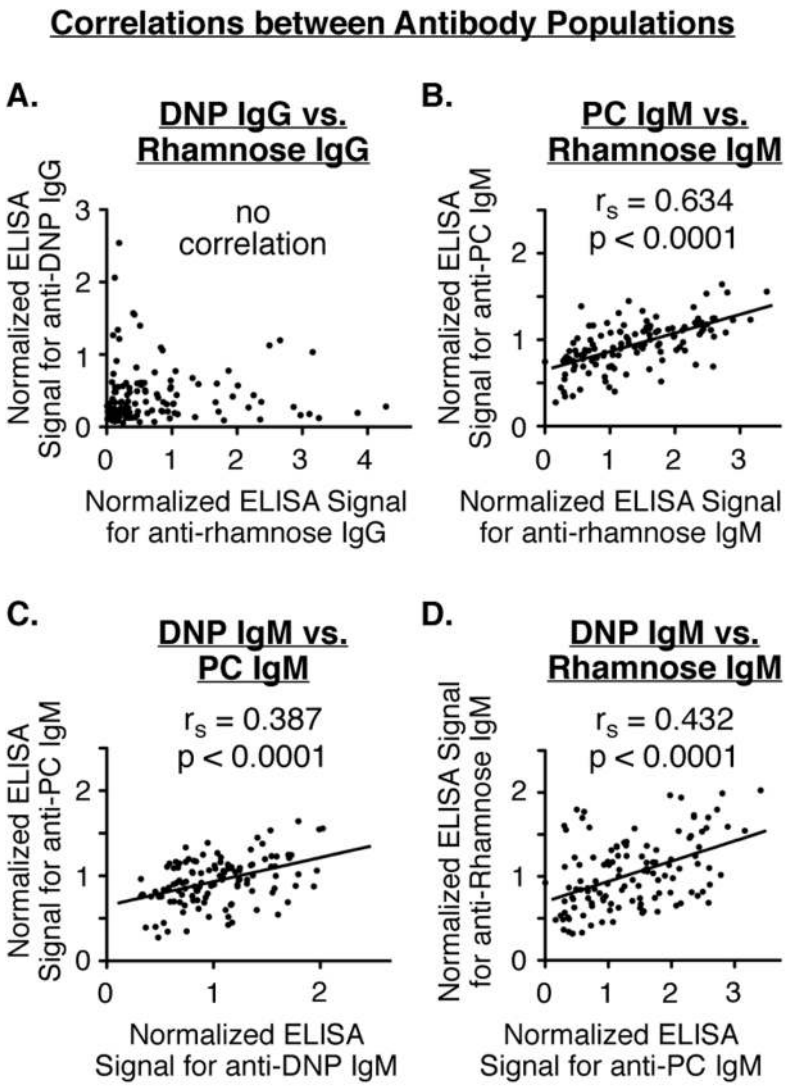

Figure 7.

Scatter plots showing correlations between data sets from Figure 6. Linear lines of best fit and Spearman rank correlation coefficients (r_s) were calculated using GraphPad Prism 5.0.

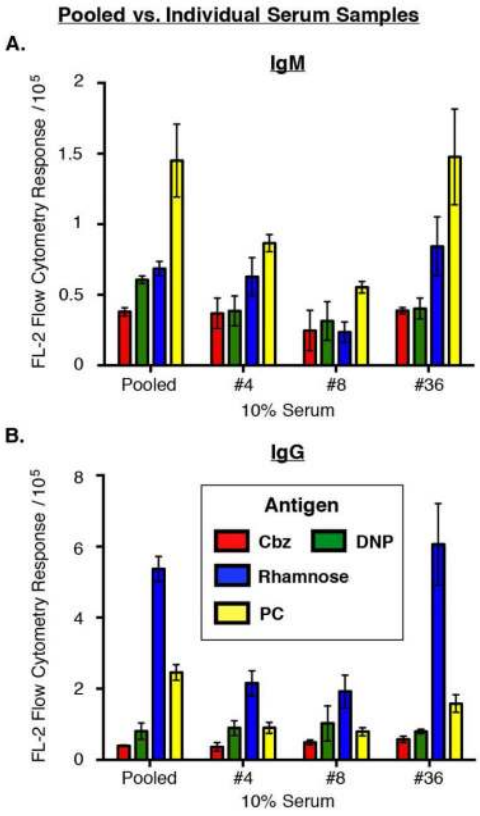

Figure 8.

Comparison of pooled serum versus three individual serum samples. (A) IgM recruitment measured by flow cytometry. (B) IgG recruitment measured by flow cytometry. Data points represent mean values of 3–8 experiments. Error bars represent 95% confidence intervals.