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Detection of human IgM and IgG antibodies by means of galactofuranose-coated and rhamnose-coated gold nanoparticles

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Abstract

Many infectious diseases lack cheap, fast, and reliable serodiagnostic tools. Recently, glycans coupled to gold nanoparticles (GNPs) have shown potential to fill this void by utilizing them as a coating in ELISA experiments. In this study, we used GNPs functionalized either with galactofuranose (Gal*f*), rhamnose (Rha*p*) or a mixture of these monosaccharides to detect the presence of IgM and IgG antibodies against these non-human carbohydrates in sera obtained from tuberculosis patients and control groups. Our findings confirm the wide abundance of anti-Rha*p* antibodies in humans and show for the first time the presence of anti-Gal*f* antibodies in human sera. This study supports further investigation of using GNP-ELISA with microbe-specific carbohydrates for serodiagnosis of infectious diseases.

Introduction

An established methodology to rapidly and specifically diagnose infection by pathogenic organisms is based on the detection of antigen-specific antibodies in body fluids by the enzyme-linked immunosorbent assay (ELISA), mostly used in combination with crude antigen extracts or recombinant proteins as specific probes. However, in many infections by bacteria, fungi, and many clinically important parasites, anti-glycan immune responses are dominant over anti-protein responses. Pathogen-specific glycan antigens presented as a dense array of multivalent structures generate high-affinity molecular interactions in the natural context and also trigger immediate immune responses, including anti-carbohydrate antibodies in the host [1].

A major bottleneck for the development of glycan-based serodetection has been that single glycan-antibody interactions are of relatively low affinity [2], but recently glycan gold nanoparticles (GNPs) have overcome this problem by presenting carbohydrates in a multivalent manner. Furthermore, they enable the coating of ELISA plates, which is not possible with monosaccharides. Thus, GNPs have shown strong potential to be used in high-throughput screening and are relatively easy and cheap to synthesize depending on the availability of the carbohydrates used [3].

For many years, galactofuranose (Gal*f*)-containing macromolecules have been a target for diagnosis of fungal diseases like aspergillosis based on antibody binding [4] [5]. Another interesting monosaccharide found in many microbes and possessing antigenic capacity is rhamnose (Rha*p*) [6] [7]. Both these monosaccharides are found in a variety of glycoconjugates present in the cell envelope of the pathogen *Mycobacterium tuberculosis (Mtb)* [8] and could thus potentially serve as antigens for antibody generation in tuberculosis (TB) patients. Diagnosis of this devastating infectious disease would benefit greatly from cheap high-throughput methods [9] and thus it seems worthwhile to explore the use of glycan-GNPs based on Gal*f* and Rha*p* in serodiagnosis experiments with sera from TB patients.

Objective

This study has two objectives. First, to assess whether antibodies in human serum can specifically recognize GNPs coated with Gal*f* and Rha*p* presented in a multivalent way.

Secondly, to determine whether a serodiagnostic screen using gold nanoparticles coated with these monosaccharides could have diagnostic value in discriminating between TB patients and control groups.

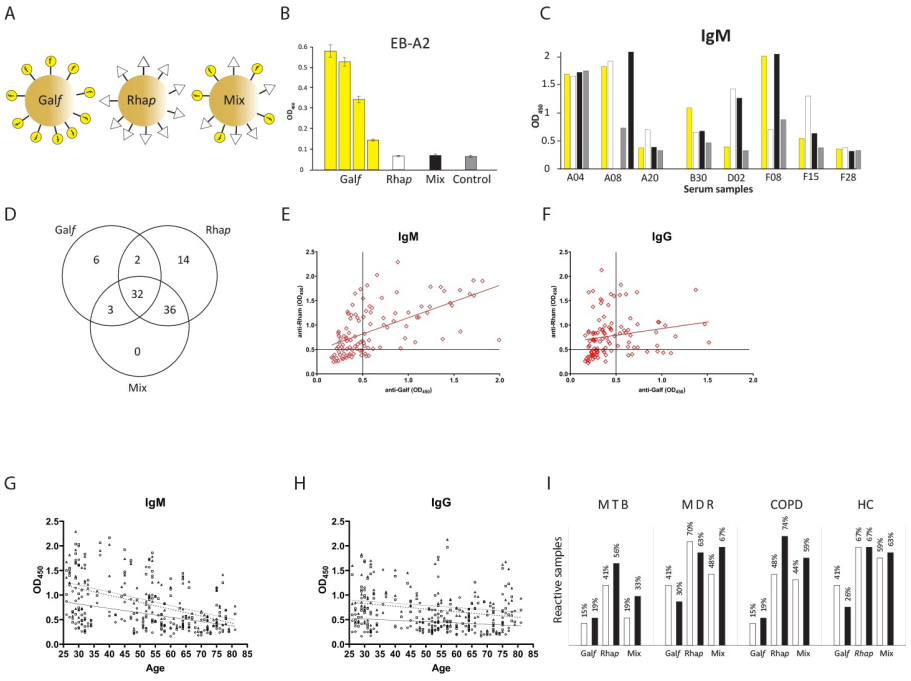

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Figure Legend

Figure 1.

(A) Galactofuranose (Gal*f*, yellow spheres) and rhamnose (Rha*p*, white triangles) monosaccharides were coupled to gold nanoparticles (large gold spheres). Mix-GNPs containing both Gal*f* and Rha*p* in a 1:1 ratio.

(B) Gal*f*- (two-fold serial dilution from 25–3.125 µg/mL), Rha*p*- (50 µg/mL), Mix- (50 µg/mL) and Control-GNPs (50 µg/mL) were used for coating Nunc-Maxisorp ELISA plates in triplicate and subsequently detected with HRP-conjugated EB-A2. One representative experiment (out of three) is shown, with error bars indicating SEM.

(C) Exemplar serum samples showing the differential presence of IgM antibodies binding to Galf- (yellow), Rhap- (white), Mix- (black) and Control-GNPs (grey). Letters in the patient codes indicate the subject group (A = tuberculosis (TB), B = multidrug-resistant TB, D = chronic obstructive pulmonary disease (COPD), F = healthy control). Bars show the average OD450 value obtained from duplicate measurements.

(D) Venn diagram showing overlap between individual sera in their reactivity against Gal*f*-, Rha*p*- and Mix-GNPs. Individual sera were scored as positive when OD450 was higher than 0.5 AND over 1.5 times the value measured for control-GNPs. Out of 108 sera, 15 did not react with any of the glycan-GNPs.

(E) Scatter plot of anti-Gal*f*-IgM values against anti-Rha*p*-IgM values of each individual subject. The line indicating the best fit and the Spearman rank correlation coefficient of 0.6638 (p <0.001) were calculated using GraphPad Prism 8.0.

(F) Scatter plot of anti-Gal*f*-IgG values against anti-Rha*p*-IgG values of each individual subject. The line indicating the best fit and the Spearman rank correlation coefficient of 0.2696 (p <0.0533) were calculated using GraphPad Prism 8.0.

(G) Age of all subjects plotted against IgM reactivity against Gal*f*- (circles and solid line), Rha*p*- (triangles and dashed line) and Mix-GNPs (squares and dotted line).

(H) Age of all subjects plotted against IgG reactivity against Gal*f*- (circles and solid line), Rha*p*- (triangles and dashed line), and Mix-GNPs (squares and dotted line).

(I) Percentage of the 4 subject groups having IgM (white bars) or IgG antibodies (black bars) against Gal*f*-, Rha*p*- or Mix-GNPs. Individual sera were scored as positive when OD450 was higher than 0.5 AND over 1.5 times the value measured for control-GNPs.

Results & Discussion

N-Hydroxysuccinimide (NHS)-functionalized GNPs were used as scaffolds to conjugate synthetic Gal*f* and Rha*p* monosaccharides functionalized at their anomeric position with a short (C_3) aliphatic amino ending spacer. GNPs with either monosaccharide or a combination of both thus generated Gal*f*-GNPs, Rha*p*-GNPs, and Mix-GNPs, respectively (Fig. 1A). Control-GNPs were prepared by reacting the 5 nm NHS-gold nanoparticles with ethanolamine. The presence of Gal*f* on the Gal*f*- and Mix-GNPs was tested in an ELISA with the rat monoclonal antibody EB-A2 that is widely used to detect circulating Gal*f*-containing galactomannan in the serum of aspergillosis patients [10] and was demonstrated previously to also recognize Gal*f*-GNPs [11]. Here, EB-A2 bound to Gal*f*-GNPs in a dose-dependent manner (25 to 3.125 µg/mL), while Rha*p*- or Control-GNPs did not show any binding up till the highest tested concentration of 50 µg/mL (Fig. 1B). Interestingly, it was previously thought that EB-A2 is only able to bind galactomannan having branches of a length of at least four β1-5 galactofuranosyl moieties [12] [13]. Although a recent glycoarray study has shown that two β1-5 galactofuranosyl moieties are sufficient to enable EB-A2 binding [14], our data suggest that other Gal*f* conformations also enable this antibody to bind. To understand the interaction between EB-A2 and Gal*f* moieties on GNPs in detail, studies, like done previously for the interaction between antibody CS35 and arabinofuranosides, could be conducted [15]. Unexpectedly, EB-A2 did not recognize the Mix-GNPs, although they also contain multiple Gal*f* moieties on their surface. Clearly, IgM and IgG antibodies in the human sera of this study were capable of binding to the Mix-GNPs, irrespective whether the sera contained antibodies that also recognized Rha*p*-GNPs or Gal*f*-GNPs alone (Fig. 1C), thereby demonstrating that the monosaccharides coupled to the GNPs are presented in a way that enables antibody binding. Taken together, these results indicate that the Gal*f*-moieties only enable binding of EB-A2 antibodies to Gal*f*-GNPs if presented in a particular manner and that the Rha*p*-moieties present on the Mix-GNPs prevent this.

Next, we used these synthesized GNPs to test the presence of antibodies recognizing Gal*f* and Rha*p* in 108 individual sera obtained from 4 different groups, i.e. patients infected with a drug-sensitive strain of Mycobacterium tuberculosis (MTB), those infected by a multi-drug resistant strain (MDR), patients suffering from chronic obstructive pulmonary disease (COPD) and healthy individuals (HC). Raw data can be found in the supplementary files S1, S2 and S3.

This experiment shows that many individuals, irrespective from their disease background, have serum IgM and IgG antibodies that can recognize Gal*f* or Rha*p* (Fig. 1C-1I). Several other studies have shown human serum antibodies against Rha*p* [1] [6] [16], however, to our knowledge, anti-Gal*f* antibodies have never been demonstrated before in human sera.

We decided to classify any serum sample that produced an optical density at 450 nm (OD450) value for a particular GNP above 0.5 and at least 1.5 times the value of reactivity against Control-GNPs as being positive against that particular GNP. So for example, samples A04 and F28 from the exemplar results shown in figure 1C were not classified as positive for either Gal*f*-, Rha*p*- or Mix-GNPs (in total 37 samples were negative for IgM and 30 for IgG). Samples with a profile similar to A08 were classified as having antibodies against all three glycan-GNPs (20 for IgM and 16 for IgG); samples similar to A20 as having antibodies against Rha*p*-GNPs only (17 for IgM and 13 for IgG); samples

like B30 as having antibodies against Gal*f* only (8 for IgM and 3 for IgG); samples like D02 as having antibodies against Rha*p*- and Mix-GNPs (24 for IgM and 39 for IgG); and samples similar to F08 as having antibodies against Gal*f*- and Mix-GNPs (2 for IgM and 4 for IgG). Just one sample (B15) was classified as only positive for having IgG against Mix-GNPs, as the signal against Rha*p*-GNPs did not pass the OD450 threshold of 0.5. Two samples (A04 and B29) were classified as positive only for IgG antibodies against Gal*f*-GNPs and Rha*p*-GNPs, but not for Mix-GNPs. In both cases, this seems to be caused by marginal differences in measured OD450 values.

In total, 27 sera resulted in value above 0.5 when incubated with the Control-GNPs and secondary detection for IgM antibodies. 5 of those had a value above 1 like shown for A04 (Fig. 1C, grey bar). In the IgG detection experiment, these numbers were much lower (9 above 0.5 and none above 1), indicating the higher specificity of the IgG isotype.

All data for both IgM and IgG can be aggregated into the Venn diagram shown in figure 1D describing the overlap between Rha*p* and Gal*f* recognition (for tables for IgM and IgG separately see Suppl. data file S4). Of the 108 individual sera tested in this study, 15 were not classified to contain either IgM or IgG antibodies reacting to any of the GNPs (14%), where 93 individuals (86%) seem to have at least one type of antibody capable of recognizing an epitope on the Gal*f*-, Rha*p*-, or Mix-GNPs (Fig. 1D). Of those, 84 individuals had antibodies that could recognize Rha*p* (90%), while 43 possessed antibodies that reacted against Gal*f* (46%).

When the levels of IgM antibodies against Galf are compared with those against Rha*p* (Fig. 1E) a significant positive correlation with a Spearman rank coefficient (rs) of 0.66 (p <0.001) is found, comparable as what has been described for correlations between anti-Rha*p* IgM and IgM antibodies recognizing other two other haptens (Jakobsche *et al.*, 2013). There was also a positive correlation between anti-Gal*f* IgG and anti-Rha*p* IgG in our study (Fig. 1F), albeit much weaker (rs = 0.27 and p = 0.053). As expected correlations for antibodies recognizing the single glycan-GNPs with the ones reacting with the Mix-GNPs are high (Suppl. data file S5).

IgM antibodies against Rha*p* (56%) were slightly less abundant than IgG antibodies against Rha*p* (65%), whereas, for anti-Gal*f* antibodies, this is reversed, with 28% of the sera having IgM antibodies against this hapten and 23% having IgG antibodies. There was no correlation between reactivity against either Rha*p* or Gal*f* based on IgM antibodies and reactivity based on IgG antibodies within individual samples (data not shown).

When the age of all tested individuals is plotted against the amount of IgM antibodies shows a negative correlation for anti-Gal*f*-GNP IgM (rs = -0.009 and p = 0.0003), anti-Rha*p*-GNP (rs = -0.014 and p <0.0001) and anti-Mix-GNP (rs = -0.014 and p <0.0001) show a negative correlation (Fig. 1G), which is in line with previous studies [1] [16] [17]. The negative correlation with age is much less pronounced for IgG antibodies (Fig. 1H), with only anti-Mix-GNPs showing a significant correlation (rs = -0.006 and p = 0.012). This is important, as the average age of the individuals of the four groups in this study differed significantly at time of sampling, with MDR (44.0 years ±13.7) and HC (40.0 years ±11.4) being relatively low and MTB (56.9 years ±11.5) and COPD (69.3 years ±8.0) being relatively high (Suppl. data file S6). When all data is clustered per patient group, it becomes immediately clear that more antibodies of both isotypes against Rha*p* and Mix were found across the 4 groups compared to anti-Gal*f* antibodies (Fig. 1I). Interestingly, the highest number of individuals with IgG antibodies against Rha*p*-GNPs (74%) was found in the COPD group, despite the high average age in that group.

Conclusions

Using only a few µL of human serum, it is possible to screen for IgM and IgG antibodies reacting with GNPs coated with individual monosaccharides or a mixture of them in an ELISA setup. A large majority of humans, irrespective of any underlying disease, have antibodies against Rha*p*, whereas a smaller percentage has antibodies against Gal*f*.

Clearly, the GNPs described in this study is not a good tool for serodiagnosis of TB, but

our approach could be helpful for follow-up studies screening sera from patients suffering from infectious diseases like TB, cryptococcosis, and aspergillosis using more specific oligosaccharides coupled to GNPs, or more comprehensive sets of different GNPs.

Limitations

One limitation of this study was the amount of serum available for doing multiple tests in a controlled manner. Ideally, one would like to test serum from individuals at multiple times. Furthermore, one would ideally have all TB patients tested to be on a similar treatment regime. However, this was not possible due to practical and logistic constraints. We had tried to include the same number of male and female in each group, as well as have individuals with similar age, however, this proved to be impossible due to the nature of the groups.

Alternative Explanations

Caution must be taken with regards to the specificity of the antibodies binding to the different GNPs. It cannot be excluded that many antibodies recognizing completely different epitopes could cross-react with the glycan-GNPs. However, such considerations could be addressed by conducting some of the experiments proposed in the Conjectures section.

Conjectures

We see the following ways forward based on the findings of this paper.

1) Screen human sera from different disease backgrounds with a more extensive panel of monosaccharides coupled to GNPs to see whether they could be predictive of a disease;

2) More specific TB oligosaccharides like arabinomannan and arabinogalactan could be used to functionalize GNPs aiming at correlating the anti-carbohydrate IgG or IgM with pathogen-exposure before and after treatment with antibiotics;

3) Couple specific oligosaccharides identified on other infectious microbes to GNPs and perform serodiagnosis experiments.

Additional Information

Methods

Preparation of glycan-coupled gold nanoparticles (GNPs)

1,2,3,5,6-Penta-*O*-acetyl-D-galactofuranose was purchased from Carbosynth. All other chemicals were purchased as reagent grade from Sigma-Aldrich and used without further purification. Gal*f*-C3-NH2 and Rha*p*-C3-NH2 were synthesized as previously described [11] and subsequently coupled to *N*-Hydroxysuccinimide (NHS)-functionalized GNPs with a diameter of 5 nm (Cytodiagnostics™) by adapting the supplier protocol designed for protein conjugation. Briefly, GNPs (1 mg) were dissolved in 100 µL of reaction buffer (pH 9) and split equally over two vials. A water solution (0.1 M) of the amino-ending glycans was mixed with the resuspension buffer (50 µL, pH 9) to reach a final glycan concentration of 25 mM, and added to the gold nanoparticle solution. The reaction was stirred for 2 h at ambient temperature, before adding ethanolamine (10 µL) for 30 min. The mixture was subsequently diluted with H_2O (300 µL) and filtered over 30 kDa MWCO cut-off centrifugal filters against H_2O (7×400 µL). The eluate from the purification steps was collected, desalted using Sephadex G-25 (GE healthcare™ PD-10 columns), and gravimetrically analyzed to estimate the loading of the glycans on the nanoparticles (~40% of loading yield for both nanoparticles). The particles were stored in a concentration of 0.5 mg/mL at 4℃.

GNP-ELISA with EB-A2 antibody

The GNP stock solutions were diluted in coating buffer (50 mM Na_2CO_3, pH 9.6) to final concentrations of 50, 25, 12.5, 6.25 and 3.125 µg/mL and subsequently used to coat wells of a NUNC MaxiSorp plate at 4℃ for 16 h. After washing with PBS (2×200 µL), 1% BSA (protease-free, Roche) in PBS was used for blocking at ambient temperature for 30 min. Subsequently, 65 µL of EB-A2-HRP (Platelia Aspergillus EIA kit, Bio-Rad) was diluted 1:3 in assay buffer (0.5% BSA) and added to the wells, after which the plate was shaken at 500 rpm for 1 h. After further washing with PBS (3×200 µL), 100 µL of TMB (3,3',5,5'-Tetramethylbenzidine) solution (Beyotime) was added. The reaction was stopped after approximately 25 min with 50 µL of 0.8 M H_2SO_4, and binding was measured at an optical density (OD) of 450 nm in a plate reader (Biotek).

Collection and handling of human sera

Serum samples (1 mL) were collected from individuals diagnosed as infected with drug-sensitive *Mtb* (MTB, patients coded A), infected with multi-drug resistant *Mtb* (MDR, patients coded B) or diagnosed with chronic obstructive pulmonary disease (COPD, patients coded D). Besides this latter, non-infected control group, sera from individuals that had come to the hospital for a routine health check and could be considered healthy controls were also collected (HC, individuals coded F). Samples were selected for inclusion in the study based on matching gender and age, although this proved to be difficult (Suppl. data file S6). In total 108 samples (27 from each group) were selected and used for subsequent antibody screening.

GNP-ELISA with human IgM/IgG antibodies

IgM/IgG testing in the human sera was performed using GNP coated immune ELISA plates. The GNP-ELISA experiments were adapted from a reported protocol (Chiodo *et al.*, 2013). 50 µL of GNP solution (12.5 µg/mL) in coating buffer (50 mM Na_2CO_3, pH 9.7) was used to coat 96-wells Nunc MaxiSorp plates for 2 h at ambient temperature, or for 16 h at 4℃. Throughout the experiment, each 96-wells plate was coated with the 4 different GNPs in a setup that enabled screening of 3 different samples from each group (A, B, D, and F) in duplicate. After discarding the GNP solutions and twice washing with 200 µL PBS (10 mM, pH 7.4), blocking was done with 200 µL of 1 % BSA (protease free, Roche) in PBS at ambient temperature for 30 min. Subsequently, the blocking solution was discarded and 100 µL of patient sera diluted 1:100 in assay buffer (0.5% BSA) were added to each well. After shaking at 500 rpm for 1 h, the wells were subsequently washed 3 times with 200 µL PBS 0.01 % v/v Tween 20. Then, 100 µL of 0.8 µg/mL of goat anti-human IgG-horse radish peroxidase (HRP, Invitrogen) or goat anti-human IgM-HRP (Invitrogen) was added. After 30 min of shaking at 500 rpm, the wells were washed 3 times with 200 µL PBS 0.01 % v/v Tween 20. Finally, 100 µL of 3,3',5,5'-Tetramethylbenzidine substrate solution (Beyotime) was added. After incubating for 6 min at ambient temperature the reaction was stopped with 50 µL of H_2SO_4 (0.8 M). The level of binding to the GNPs was measured at an optical density of 450 nm in a plate reader (Biotek).

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Ethics Statement

Written informed consent was obtained from all study participants according to regulations in the Tianjin Haihe Hospital. The Ethics Committee of Xi'an Jiaotong-Liverpool

University provided written approval (document EXT 16-03-01) prior to conducting the study, judging that all methods in the study met relevant ethical guidelines and regulations according to the Declaration of Helsinki.

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