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Diagnostic GPI

Diagnosis of Toxoplasmosis Using a Synthetic Glycosylphosphatidylinositol Glycan**

*Sebastian Götze, Nahid Azzouz, Yu-Hsuan Tsai, Uwe Groß, Anika Reinhardt, Chakkumkal Anish, Peter H. Seeberger, and Daniel Varón Silva**

Abstract: *Around 2 billion people worldwide are infected with the apicomplexan parasite Toxoplasma gondii which induces a variety of medical conditions. For example, primary infection during pregnancy can result in fetal death or mental retardation of the child. Diagnosis of acute infections in pregnant women is challenging but crucially important as the drugs used to treat T. gondii infections are potentially harmful to the unborn child. Better, faster, more reliable, and cheaper means of diagnosis by using defined antigens for accurate serological tests are highly desirable. Synthetic pathogen-specific glycosylphosphatidylinositol (GPI) glycan antigens are diagnostic markers and have been used to distinguish between toxoplasmosis disease states using human sera.*

The apicomplexan *T. gondii* is a parasite that is capable of infecting all warm-blooded animals.[1] Infection occurs through the ingestion of contaminated raw meat or oocysts shed by cats, the parasite's primary host.[2] Although *T. gondii* infections are the third leading cause of food-borne infections requiring hospitalization in the USA,[3] it is not perceived as a major public health problem in developed countries, owing to its usually benign etiopathology.[4] However, in immunocompromised individuals, latent toxoplasmosis can lead to dangerous medical conditions such as toxoplasmic encephalitis, a severe inflammation of the brain and a leading cause of death in AIDS patients.[5] Primary infection during pregnancy can cause transmission of the parasite from the mother to the unborn child, leading to mental and physical retardation and fetal death.[6] Congenital toxoplasmosis can be prevented by stopping parasite transmission from mother to fetus using potentially harmful chemotherapy. Thus, false-positive diagnoses of the disease are to be avoided. Currently, diagnosis of acute toxoplasmosis relies on serological detection of IgG and IgM antibodies as well as determination of IgG avidity against *T. gondii* antigens. The IgM titer has the greatest diagnostic value, as the absence of IgM antibodies can rule out a recently acquired infection with greater certainty. However, commercially available test kits used by nonreference laboratories are known to generate high rates (up to 60%) of false-positive test results.[7] Therefore, the identification of antigens that are suitable for the development of reliable diagnostic tools is important.

Glycosylphosphatidylinositols (GPIs) are a class of complex molecules that are present in high abundance (about 10^6 copies per cell) on the cell surface of *T. gondii*.[8] These glycolipids are found in all eukaryotic organisms and share the conserved glycan core structure α-Man-(1→2)-α-Man-(1→6)-α-Man-(1→4)-α-GlcNH$_2$-(1→6)-*myo*-Ino (Figure 1).[9] *T. gondii* uses GPIs to anchor enzymes and other surface proteins via their C-terminus to the extracellular side of the plasma membrane. Two main GPI glycoforms that are *T. gondii* specific contain an additional branch connected to the O4-position of Man I. Whereas GPI **1** bears an α-Glc-(1→4)-β-GalNAc side chain, GPI **2** lacks the glucose moiety.[10] Molecules **1** and **2** also differ in their function. While GPI **2** serves as a membrane anchor for surface antigens and enzymes, GPI **1** occurs free on the cell surface without being covalently attached to any protein.[11] In particular, GPI **1** is an immunologically active compound that induces an early IgM response in humans after infection with *T. gondii*.[12] We hypothesized that the phosphoglycans of GPIs **1** and **2**, as well as substructures and biochemical intermediates, display potential diagnostic markers that may enable the differentiation between acute and latent toxoplasmosis.[13]

The isolation of homogeneous GPIs from parasites is challenging and does not give access to GPI derivatives or substructures. We employed chemical synthesis to obtain GPIs[14] of defined structure and high purity that are suitable for biochemical assays and medical applications.[15] This approach also excludes the possibility of false-positive test results due to cross-reactivity against impurities in *T. gondii* isolates.[16] In order to elaborate diagnostic tools based on carbohydrate microarrays[17] of GPI-glycans, an orthogonal handle that ensures regioselective covalent attachment to a solid support was required. To preserve the free amine groups of GPIs, a thiol was selected as a nucleophilic orthogonal functionality. Therefore, a linear alkane thiol

[*] Dr. S. Götze, Dr. N. Azzouz, Dr. Y.-H. Tsai, A. Reinhardt, Dr. C. Anish, Prof. Dr. P. H. Seeberger, Dr. D. Varón Silva
Department of Biomolecular Systems
Max Planck Institute of Colloids and Interfaces
Am Mühlenberg 1, 14424 Potsdam (Germany)
and
Institute of Chemistry and Biochemistry
Freie Universität Berlin
Arnimallee 22, 14195 Berlin (Germany)
E-mail: daniel.varon@mpikg.mpg.de
Prof. Dr. U. Groß
Nationales Konsiliarlabor Toxoplasma und Institut für Medizinische Mikrobiologie, Universitätsmedizin Göttingen (Germany)

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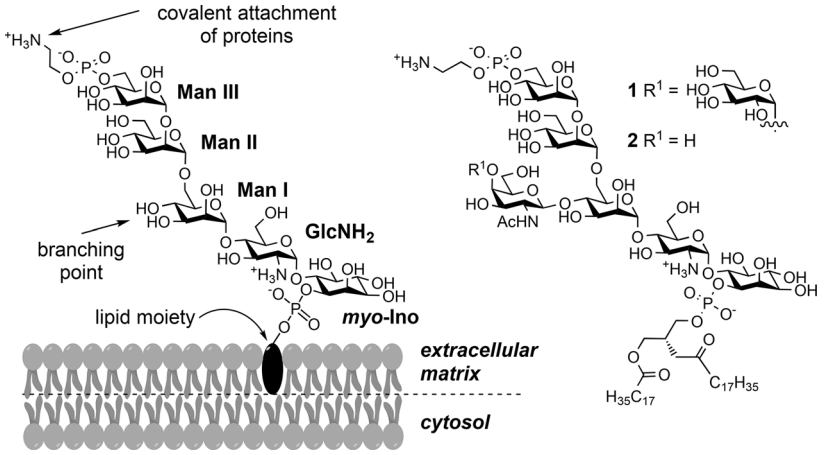
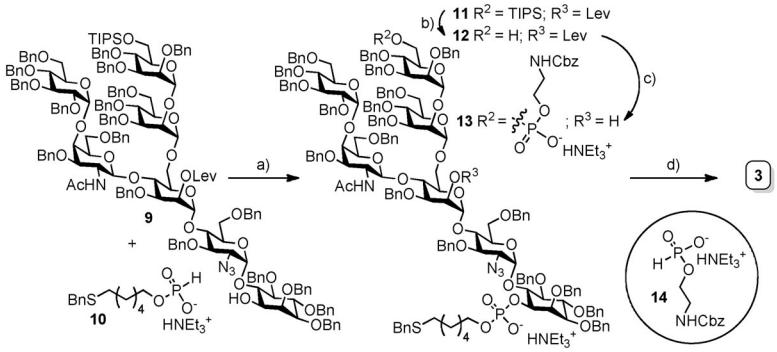


Figure 1. Conserved pseudopentasaccharide core structure of GPIs and *T. gondii* specific glycolipids **1** and **2**. $GlcNH_2$ = D-glucosamine, Ino = inositol, Man = D-mannose.

phosphodiester was installed at the O1-position of *myo*-Ino instead of the naturally occurring lipid moiety to provide conjugation-ready phosphoglycans **3**–**6** (Figure 2).[18]

To investigate the immune response against the carbohydrate portion of **1** and **2** upon infection with *T. gondii* and determine the immunodominant epitopes, such as side-branch glycosylation or phosphorylation status, the glycans **3** and **4**, as well as a set of closely related sugars, were synthesized. Glycan **5** represents a biosynthetic precursor of GPI **2** that is not phosphorylated at the O6-position of Man III.[10] Trisphosphorylated compound **6** reflects a minimal structure of mammalian GPIs, which possess an additional phosphoethanolamine and a GalNAc branch at the O2- and O4- positions of Man I respectively.[15b]

Access to the complex GPI molecules was based on the convergent route for the synthesis of these glycolipids and their derivatives we had developed earlier.[19] To illustrate the syntheses of glycans **3**–**6**[20] the preparation of **3** is shown in detail (Scheme 1). The synthesis commenced with the phosphitylation of alcohol **9**[19] by an in situ generated mixed anhydride of *H*-phosphonate **10**[18] and pivaloyl chloride. Subsequent oxidation of the resulting *H*-phosphonate using iodine in wet pyridine furnished phosphate salt **11** in 87% yield. Acidic hydrolysis of the silyl ether gave access to primary alcohol **12**. A one-pot procedure yielded bisphosphate **13** through phosphitylation of **12** using *H*-phosphonate **14**[14d] followed by oxidation and final hydrazinolysis of the levulinic ester in 76% yield. For global deprotection of the remaining benzyl ether units **13** was submitted to Birch reduction conditions to furnish carbohydrate antigen **3** in 55% yield.[15g]



Scheme 1. Synthesis of phosphoglycan **3**. Reagents and conditions: a) 1. **10**, PivCl, Py; 2. I_2, H_2O, 87%; b) $Sc(OTf)_3$, MeCN, H_2O, 50 °C, 76%; c) 1. **14**, PivCl, Py; 2. I_2, H_2O; 3. hydrazine, 76%; d) $NH_{3(l)}$, THF, $Na_{(s)}$, −78 °C, *t*BuOH, 55%. Bn = benzyl, Cbz = carboxybenzyl, Lev = levulinyl, Piv = pivaloyl, Py = pyridine, Tf = triflyl, TIPS = triisopropylsilyl.

To complete the collection of synthetic GPI glycans related to toxoplasmosis antigens, the substructures **15**–**17** (Figure 3A) displaying the side branch of GPI **3** were also prepared according to the general synthetic strategy.[20] Linear glycans **18** and **19**,[18] which mirror GPI anchors found on the apicomplexan parasites *Plasmodium falciparum* and *Cryptosporidium parvum*,[21] as well as pseudodisaccharide **20**[18] (Figure 3B) were synthesized and placed on the GPI-glycan arrays to examine a possible immune response against the conserved backbone. Mannan-related tetramannoside **21**[22] (Figure 3C) served as a control carbohydrate as it is not related to the GPIs of *T. gondii* but is found in certain types of mycobacteria[23] and fungi.[24]

All carbohydrates bearing either a single amine or thiol linker were printed on epoxide-modified glass slides and covalently immobilized on the surface.[25] The resulting carbo-

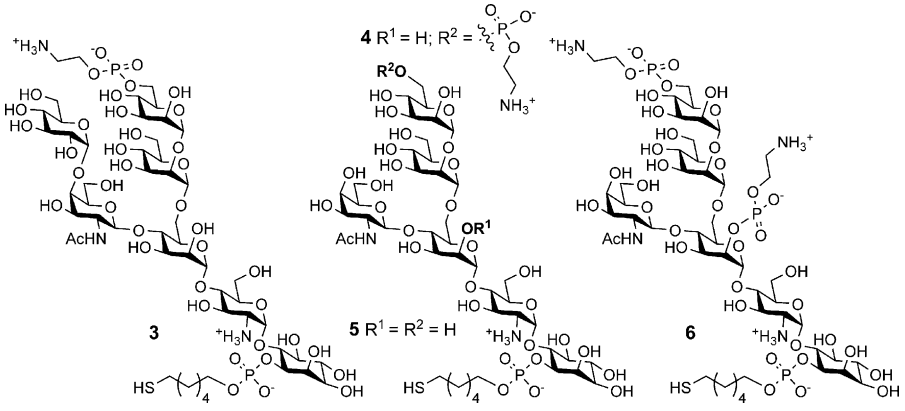


Figure 2. GPI-glycans **3**–**5** resemble the carbohydrate moieties of *T. gondii* GPIs. Compound **6** represents the minimal structure of all mammalian GPIs.

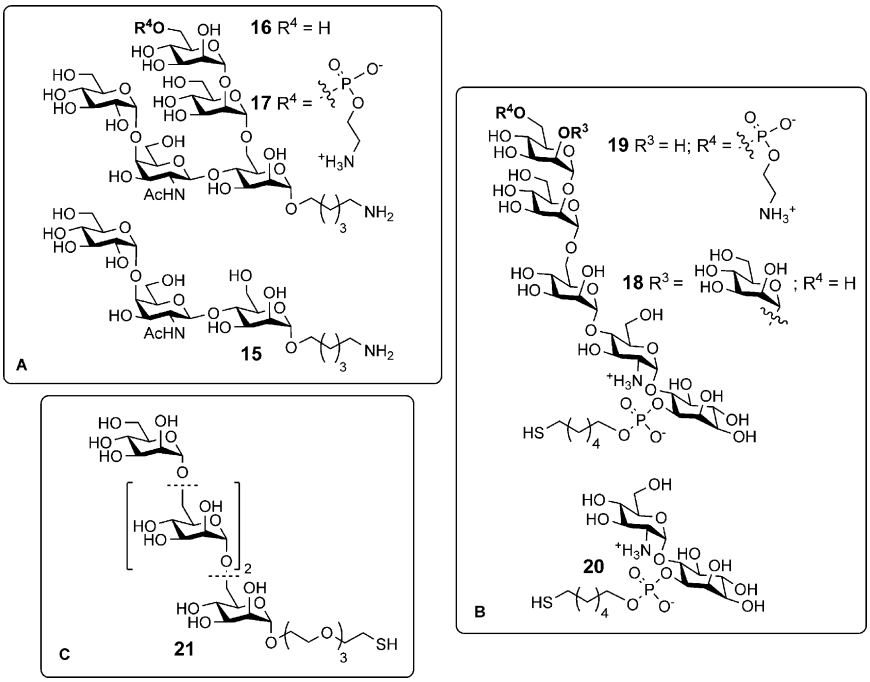


Figure 3. Carbohydrate structures evaluated as markers for the diagnosis of toxoplasmosis. A) Substructures **15**–**17** represent the side branch of GPI **3**. B) Linear phosphoglycans **18**–**20** contain conserved features of GPIs. C) Mannan substructure **21** is present in the cell wall of pathogenic yeast.

hydrate microarrays were incubated with reference sera of patients suffering from acute or latent toxoplasmosis as well as of seronegative individuals utilizing a highly reproducible protocol for the detection of anti-carbohydrate antibodies in humans.[26]

The screening results (Figure 4) showed that all sera from noninfected patients[27] contained undetectable or low levels of IgG and IgM antibodies directed against the printed GPIs or their substructures (Figure 4A). Some healthy individuals exhibited IgG and IgM antibodies that bound to tetramannoside **21** in detectable concentrations; these are not attributed to an infection with *T. gondii*, but possibly other pathogens.[28]

In contrast, all sera from patients diagnosed with acute toxoplasmosis showed high levels of IgG and IgM antibodies recognizing the full glycan structure **3** (Figure 4B). In addition IgG and IgM antibodies directed against substructures **15**–**17** of GPI **1** were also found in almost all samples from acutely infected persons exhibiting signal intensities comparable to those obtained for phosphoglycan **3**. Most acute-toxoplasmosis samples contained IgG and IgM antibodies that bind phosphoglycan **4**, but the intensity of the signals was usually lower compared to those generated by the α-Glc containing compounds **3**, **15**, **16**, and **17**. IgG and IgM antibodies binding to linear GPI **19** and monophosphorylated GPI-glycan **5** were detected in some sera of acutely infected patients. Antibodies recognizing the *P. falciparum* GPI-glycan structure **18** were not observed in any acutely infected patients. Interestingly, individual sera also contained IgM but no IgG antibodies directed against pseudodisaccharide **20**.

As anticipated, samples from latently infected patients showed an IgG antibody binding pattern and signal intensities that are comparable to the analyses of sera from acutely infected humans (Figure 4C). In contrast, IgM levels against all printed structures were considerably reduced.

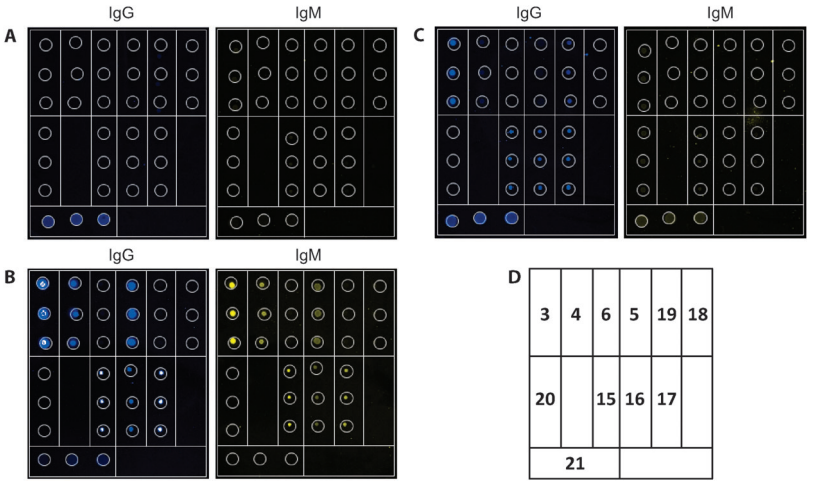


Figure 4. Representative scans of a carbohydrate microarray incubated with generic serum samples (dilution 1:15) from patients having A) no infection, B) acute toxoplasmosis, and C) latent toxoplasmosis (printing pattern is superimposed). D) Scheme describing the positions of the compounds on the microarray.

These results are in accordance with reports describing that the immune response against GPIs in *T. gondii* infected humans is mainly directed against the α-Glc-containing glycolipid **1** and not GPI anchor **2**, which indicates that the side-branch motif in this molecule is an important antigenic epitope.[11] None of the tested samples contained antibodies that bind to mammalian glycan **6**, suggesting that the adaptive immune system is able to distinguish self from non-self GPI structures through the additional phosphoethanolamine unit at Man I.

As antibodies against GPI-glycan **3** were detected in all infected samples, this structure was evaluated as a diagnostic marker for toxoplasmosis (Figure 5). A statistical analysis showed that mean IgG as well as IgM serum antibody levels against saccharide **3** are significantly increased during the acute phase of the infection. While the average concentration of IgG in the blood does not

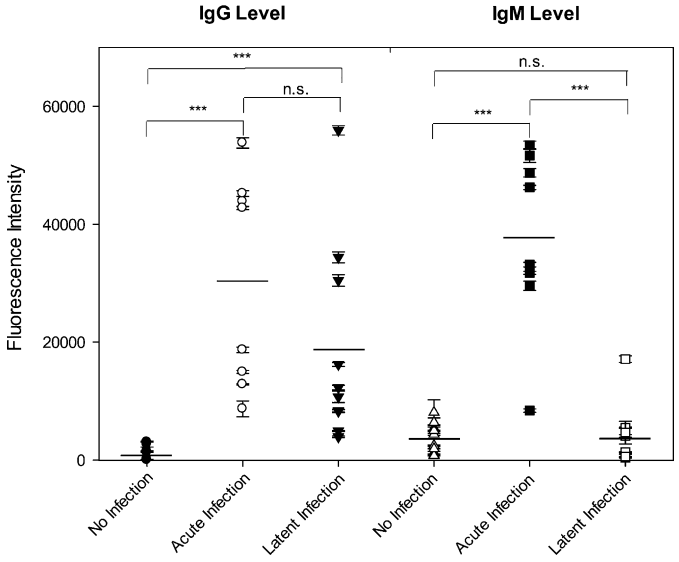


Figure 5. Antibody levels determined by microarray analysis against antigen **3** in sera samples (dilution 1:15) from different toxoplasmosis cohorts including standard deviation for every data point (n.s. = not significant; *** = $p < 0.005$, calculated using the Mann–Whitney rank sum test). Description of cohorts: no infection ($n = 10$); acute infection ($n = 8$); latent infection ($n = 10$). Black bars represent mean antibody levels.

considerably decrease after the acute phase, IgM levels drop to values that are in most cases comparable to those of seronegative individuals.

Antigen **3** emerged as a suitable biomarker for the diagnosis of different stages of toxoplasmosis. The IgG level against **3** can be used to distinguish noninfected from *T. gondii* infected humans whereas the concentration of IgM antibodies binding the same carbohydrate may serve to differentiate latent and acute toxoplasmosis.[29]

In conclusion we synthesized GPI-glycans found on the surface of parasite and mammalian cells. Parasite-specific carbohydrates presented on glycan arrays enabled a detailed investigation of the immune response against GPIs upon natural infection with *T. gondii* in humans. Screening of human sera revealed that only the full phosphoglycan of GPI **1** displays a valuable antigen recognized by all infected serum samples tested. Analysis of GPI **3** specific IgG and IgM levels allowed for distinction between infected and noninfected individuals. Thus, parallel detection of IgG and IgM directed against the single synthetic cell-surface antigen, GPI **3**, constitutes a suitable strategy for the development of a faster and more-specific diagnostic tool to discriminate between latent and acute toxoplasmosis.

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