
**USE OF SYNTHETIC CARBOHYDRATES
AS VACCINE COMPONENTS AND BIOMEDICAL
RESEARCH TOOLS**

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TABLE OF CONTENTS

Acknowledgements.....	iii
Summary – Zusammenfassung.....	iv
Chapter 1: Introduction.....	1
1.1 Synthetic Oligosaccharides for Medical Applications.....	1
1.2 Glycosylphosphatidylinositol (GPI) Anchors in Malaria Pathogenesis and Immunity.....	2
1.2.1 <i>Plasmodium</i> and Malaria.....	2
1.2.2 Role of GPI in Malaria Pathogenesis.....	5
1.2.3 Structure, Recognition and Processing of <i>P. falciparum</i> GPIs.....	6
1.2.4 GPI-anchored proteins of <i>P. falciparum</i>	8
1.2.5 Immunity to <i>P. falciparum</i> GPIs.....	9
1.2.6 Prospects for Anti-GPI Vaccination.....	10
1.3 Anthrose for Anthrax Detection.....	11
1.3.1 <i>Bacillus anthracis</i> and Anthrax.....	11
1.3.2 Anthrax in Humans.....	13
1.3.3 Anthrax as Biological Weapon: Need for Rapid-Detection Methods.....	15
1.3.4 Unique Anthrose carried by the Exosporium Glycoprotein BclA.....	16
1.4 References.....	18
Chapter 2: Goal and Objectives.....	22
2.1 Goal.....	22
2.2 Objectives.....	22
Chapter 3: Synthetic GPI array to study antitoxic malaria response.....	23
Chapter 4: Synthetic glycosylphosphatidylinositol microarray reveal differential antibody levels and fine-specificities in children with mild and severe malaria.....	38
Chapter 5: Functional activities of monoclonal antibodies raised against synthetic <i>Plasmodium falciparum</i> Glycosylphosphatidylinositol glycans.....	57

Table of Contents

Chapter 6:	Anti-Carbohydrate Antibodies for the Detection of Anthrax Spores.....	80
Chapter 7:	Immuno-detection of Anthrose Containing Tetrasaccharide in the Exosporium of <i>Bacillus anthracis</i> and <i>Bacillus cereus</i> Strains.....	91
Chapter 8:	General Discussion and Conclusions.....	121
8.1	General Discussion.....	121
8.1.1	Malaria Control, a Changing Concept from Prevalence-based to Disease-based Vaccines.....	121
8.1.2	A Dualistic Approach to study the Relevance of Anti-GPI Immune Response for Protection against Malaria.....	123
8.1.3	Lack of Reliable Surrogate Markers of Protection along the Malaria Vaccine Development Path.....	125
8.1.4	Synthetic Polysaccharide Antigens need Enhancement for Immunogenicity in Infants.....	127
8.1.5	mAbs serve as “All-Rounder” Tools in Infectious Disease Research, Therapy and Diagnostics.....	128
8.2	Conclusions.....	132
8.3	References.....	133
Appendix:	Parasite density-dependent regulation of a CCCH-type zinc finger protein in <i>Plasmodium falciparum</i> asexual blood stages.....	136
Curriculum Vitae.....		160

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SUMMARY – ZUSAMMENFASSUNG**Characterization of GPI-specific antibody responses with carbohydrate microarrays.**

Plasmodium falciparum malaria affects about 500 million people worldwide and is responsible for approximately 2.5 million deaths per year. Drug resistance is a growing problem at a time when there is still no effective vaccine. Glycosylphosphatidylinositol (GPI) is the major anchor for membrane-associated proteins of *P. falciparum* and there is growing evidence that this glycolipid plays a major role as a toxin in the pathology of malaria. It has been postulated that antibodies against plasmodial GPI mediate anti-toxic and anti-disease immunity against malaria and that GPI could be a suitable component of a malaria vaccine. Lack of a reliable method for the characterization of anti-GPI antibodies has made it difficult to generate strong supportive data for this hypothesis. We have established carbohydrate microarrays based on synthetic *P. falciparum* GPI glycans to study antibody responses to different structural elements of this complex antigen. Results demonstrated, that only part of the total anti-GPI response in individuals from malaria endemic regions is associated with exposure to malaria. Microarray-based epitope analyses with truncated GPIs demonstrated differences in the fine specificity of anti-GPI antibodies in malaria-exposed and non-exposed populations and showed evidence for a correlation between the presence of anti-GPI antibodies with a certain fine specificity and resistance to severe malaria. The GPI microarray was also a valuable screening platform for the selection of GPI specific B cell hybridomas. The monoclonal antibodies (mAbs) generated were used to evaluate biological activities of anti-GPI antibodies in functional assays. These unique mAbs represent new research tools that will facilitate characterisation of the GPI-anchored proteome of protozoan parasites.

Use of synthetic oligosaccharide to elicit *Bacillus anthracis* specific antibodies. Anthrax is an acute zoonotic disease caused by the spore-forming bacterium *Bacillus anthracis*. The recent use of *B. anthracis* spores as a biological weapon has stressed the need for efficient vaccines and detection systems. The similarity of spore cell surface antigens to other bacteria of the *B. cereus* group has made it difficult to create selective antibody-based detection systems. On the surface of *B. anthracis* spores a tetrasaccharide containing a novel monosaccharide (anthrose) has been discovered recently. Since anthrose was not found in spores of related strains of bacteria, we envisioned the detection of *B. anthracis* spores based on antibodies against anthrose-containing polysaccharides. Carbohydrate-protein conjugates containing the synthetic tetrasaccharide, an anthrose-rhamnose disaccharide or anthrose alone were employed to immunize mice. All three formulations were immunogenic and elicited IgG responses with different fine specificities. All sera and mAbs derived from tetrasaccharide immunized mice cross-reacted with spores of a panel of virulent *B. anthracis* strains, but also with some of the *B. cereus* strains tested. Although not strictly specific for *B. anthracis* spores, antibodies against the tetrasaccharide may have potential as immuno-capturing components for a highly sensitive spore detection system.

Charakterisierung von GPI-spezifischen Antikörper-Antworten mit Kohlenhydrat-Microarrays. *Plasmodium falciparum* Malaria betrifft weltweit etwa 500 Millionen Menschen und ist verantwortlich für zirka 2.5 Millionen Todesfälle pro Jahr. Medikamentenresistenz ist ein wachsendes Problem und derzeit gibt es noch keine wirksame Impfung. Membranassoziierte Proteine von *P. falciparum* werden hauptsächlich durch Glykosylphosphatidylinositol (GPI) verankert und vieles deutet darauf hin, dass dieses Glykolipid eine bedeutende Rolle als Toxin in der Malariopathologie spielt. Es wurde postuliert, dass GPI-spezifische Antikörper antitoxische- und antikrankheits- Immunität gegen Malaria vermitteln und dass GPI eine geeignete Komponente eines Malaria Impfstoffes sein könnte. Das Fehlen einer verlässlichen Methode zur Charakterisierung von anti-GPI Antikörpern hat die Validierung dieser Hypothese erschwert. Wir haben Kohlenhydrat-Microarrays basierend auf synthetischen *P. falciparum* GPI Glykanen etabliert, um Antikörperantworten gegen die verschiedenen strukturellen Elemente dieses komplexen Antigens zu untersuchen. Die Resultate zeigten, dass nur ein Teil der gesamten Anti-GPI Antwort von Individuen aus Malaria endemischen Regionen mit Malariaexposition assoziiert ist. Microarray basierte Epitopenanalysen mit verkürzten GPIs demonstrierten Unterschiede in der Feinspezifität von anti-GPI Antikörpern bei malariaexponierten und nicht exponierten Populationen. Die Analysen lieferten Evidenz für eine Korrelation zwischen der Präsenz von anti-GPI Antikörpern einer bestimmten Feinspezifität und Resistenz gegen schwere Malaria. Der GPI Microarray fungierte auch als Plattform für die Selektion von GPI-spezifischen B Zell Hybridomen. Die generierten monoklonalen Antikörper (mAk) wurden verwendet um biologische Aktivitäten von anti-GPI Antikörpern in funktionellen Untersuchungen zu evaluieren. Diese mAk repräsentieren neue Forschungsinstrumente, welche die Charakterisierung des GPI verankerten Proteoms von parasitären Protozoen erleichtern werden.

Anwendung von synthetischen Oligosacchariden zur Herstellung von *Bacillus anthracis* spezifischen Antikörpern. Anthrax ist eine akute Zoonose verursacht durch sporenbildende *B. anthracis* Bakterien. Da *B. anthracis* Sporen als biologische Waffe benutzt wurden, werden effiziente Vakzine und Detektionsmethoden dringend benötigt. Die Ähnlichkeit von Sporenoberflächenantigenen zu anderen Bakterien der *B. cereus* Gruppe hat die Entwicklung von selektiven antikörperbasierenden Detektionssystemen erschwert. An der Oberfläche von *B. anthracis* Sporen wurde kürzlich ein Tetrasaccharid mit einem neuen Monosaccharid (Anthrose) entdeckt. Da Anthrose in Sporen von eng verwandten Bakterien nicht gefunden wurde, haben wir die Detektion von *B. anthracis* Sporen basierend auf Antikörpern gegen synthetische, anthroshaltige Polysaccharide anvisiert. Kohlenhydrat-Protein Konjugate mit dem Tetrasaccharid, einem Anthrose-Rhamnose Disaccharid und Anthrose alleine wurden verwendet, um Mäuse zu immunisieren. Alle drei Formulierungen waren immunogen und induzierten IgG Antworten mit unterschiedlichen Feinspezifitäten. Alle Seren und mAk, abgeleitet von einer Tetrasaccharid immunisierten Maus, kreuzreagierten mit Sporen von virulenten *B. anthracis* Stämmen, aber auch mit einigen getesteten *B. cereus* Stämmen. Obwohl Antikörper gegen das Tetrasaccharid nicht strikt spezifisch für *B. anthracis* Sporen sind, haben sie Potenzial als immunologische Komponente für ein hoch sensitives Sporen-Detektionssystem.

CHAPTER 1: INTRODUCTION

1.1 Synthetic Oligosaccharides for Medical Applications

While nucleic acids and proteins are linear assemblies, carbohydrates are a highly complex and diverse class of biopolymers commonly found in nature as glycoconjugates such as glycoproteins and glycolipids (1). Oligosaccharide chains (glycans) are known to mediate many important biological functions. These include viral entry, signal transduction, inflammation, cell-cell interactions, pathogen-host interactions, fertility, and development (2-9). All cells in nature are covered with a dense coating of glycans that is important for biological processes, but also for the binding of pathogens. Glycans may undergo cycles of evolutionary changes in which long-lived hosts may evade the more rapidly evolving pathogens that infect them by changing their glycan expression pattern, without compromising their own survival (10-12). This may explain the large structural variations of glycans in nature, which contribute to biological diversity and perhaps even speciation (12).

Our understanding of glycoconjugates is lagging behind advances in genomics and proteomics. Rapid advances in the field of glycomics have been hindered by the lack of general sequencing methods and difficulties in the isolation of oligosaccharides in pure form. But recently improved protocols for solution-phase oligosaccharide synthesis (13-15), as well as the use of automated carbohydrate assembly methods (1), have provided access to quantities of pure oligosaccharides sufficient for research applications. Synthetic carbohydrates allowed the development of novel tools that provide a molecular picture of biological processes involving carbohydrates. These tools include: monovalent fluorescent conjugates, neoglycoproteins, multivalent quantum-dot conjugates, affinitytagged saccharides, derivatized magnetic particles and latex microspheres, sepharose affinity resins, carbohydrate microarrays, and surface-plasmon resonance to probe carbohydrate-protein interactions (9). These techniques are now being applied for the development of carbohydrate-based diagnostics, vaccines and therapeutics.

Glycan microarrays, for example, are a low-cost platform to screen interactions involving carbohydrates in a high throughput manner. The chip-based format offers important advantages over conventional methods. These include the ability to screen several thousand binding events in parallel and the fact that a minimal amount of analyte and ligand are required. Assay miniaturization is particularly suitable for glycomics, as access to pure oligosaccharides is often the limiting factor (1, 9). The first carbohydrate arrays consisted of a variety of sugars deposited on a slide and were used to profile the glycan-binding specificity of fluorescently-labelled anti-carbohydrate monoclonal antibodies, lectins and bacterial toxins (16-21). Carbohydrate arrays also found applications in diagnostics; the GloboH hexasaccharide cancer marker and analogs were

arrayed and used to test monoclonal antibodies and patient sera for GoboH-specific binding (22). A potentially neutralizing antibody, 2G12, protects against viral challenge in animal models of HIV infection, by binding terminal Man α 1-2Man residues on gp120 (23). Carbohydrate arrays have been developed to characterize the affinity and structural specificity of 2G12 mannose recognition compared with other mannose-binding or gp120-binding proteins to develop a carbohydrate template for HIV vaccine design (18, 24-25).

In addition to serving as tools, carbohydrates also hold great potential as vaccines. The cell surfaces of pathogens exhibit oligosaccharides that are often distinct from those of their hosts. Specific types of glycoconjugate are often more highly expressed on the surface of tumours than on normal cells (26). Such cell-surface carbohydrate structures may serve as basis for carbohydrate-based detection systems and vaccines (27). Several antibacterial vaccines based on purified capsular polysaccharides or on neoglycoconjugates are now commercially available, such as vaccines against *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Haemophilus influenzae* type b and *Salmonella typhi* (28). Synthetic access to oligosaccharides of infectious agents that are difficult to culture and isolate (such as *Plasmodium falciparum* and *Bacillus anthracis*) may lead to the development of new synthetic carbohydrate vaccines.

Glycomics has just gone beyond the initial proof-of-principle studies for diagnostics and therapeutic candidates. The knowledge gained from glycomics will become as important as a basis for the pharmaceutical industry as that discovered in the fields of genomics and proteomics (27).

1.2 Glycosylphosphatidylinositol (GPI) Anchors in Malaria Pathogenesis and Immunity

1.2.1 *Plasmodium* and Malaria

Malaria is transmitted to vertebrate hosts, such as rodents, monkeys and humans, by the bite of female *Anopheles* mosquitoes that are infected with protozoan parasites of the genus *Plasmodium*. *Plasmodium* belongs to the phylum of the *Apicomplexa* that contains other human and veterinary pathogens like *Toxoplasma*, *Cryptosporidium*, *Eimeria*, *Babesia* and *Theileria*. The inoculated sporozoite stage is transient and causes no pathology. Within a few minutes, sporozoites infect liver cells and undergo a period of intracellular replication, which is also clinically silent. After schizogony the parasite initiates blood-stage infection, which is responsible for the major symptoms and pathology of malaria, the most serious being anaemia and cerebral malaria (Fig. 1) (29). Four different species cause human malaria, *Plasmodium falciparum* and *Plasmodium vivax* accounting for the majority of morbidity and mortality (30). In areas of high malaria transmission, the burden of disease is borne by infants and young children. Overall patterns of disease depend

markedly on the age and the previous immunological experience of the host (31). Adaptive immune responses in the host limit the clinical impact of infection and provide partial, but incomplete, protection against pathogen replication; however, these complex immunological reactions can also contribute to disease and fatalities (29).

Malaria is possibly the most serious infectious disease of humans, infecting 5–10% of the world's population, with 300–600 million clinical cases and more than 2 million deaths annually. Moreover, malaria is a major social and economic burden in endemic areas. There are multiple channels by which malaria impedes development, including effects on fertility, population growth, saving and investment, worker productivity, absence, premature mortality and medical costs (32). Widespread and increasing drug and insecticide resistance has exacerbated the situation, undermining the effectiveness of malaria control methods that depend on chemotherapy and vector control, respectively (33). Therefore, new approaches to combat malaria, such as vaccine development and/or discovery of novel therapeutic agents, are urgently needed (34-35).

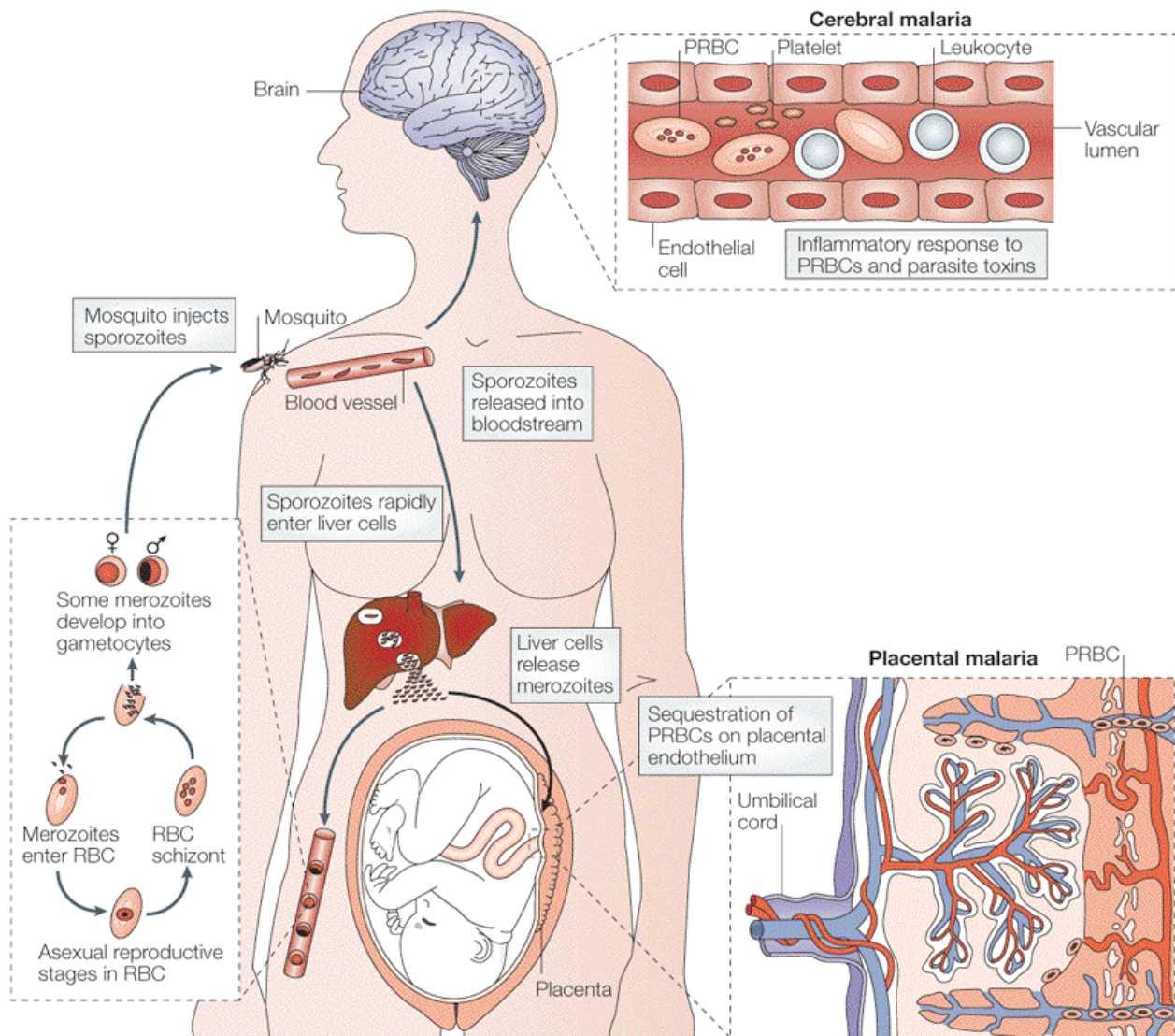


Figure 1. The life cycle of *Plasmodium falciparum*. The complex live cycle of *P. falciparum* alternating between a female mosquito and the human host starts when sporozoites are injected during the bite of an infected mosquito. The sporozoites invade within a few minutes hepatocytes and after schizogony thousands of merozoites capable of invading red blood cells are released into the blood stream. Ring, trophozoite, schizont and merozoite stages are distinguished during the asexual blood stage developmental cycle. Maturing parasites produce adherent ligands allowing binding to receptors expressed by endothelial cells of blood vessels in organs such as the brain, lungs and placenta. After 48 hours, the parasitized red blood cells (PRBCs) rupture and release more merozoites. The presence of the parasite and the invasion of red blood cells might not be sufficient to account for disease; instead, the release of bioactive parasite molecules and an inappropriately regulated host immune response could be the main causes of fatal pathogenesis, which occurs in only a minority of patients. Some merozoites differentiate into gametocytes, which, when taken up by another feeding mosquito, perpetuate the sexual cycle in the insect (29).

1.2.2 Role of GPI in Malaria Pathogenesis

Binding of diverse endothelial cell-expressed receptors by *P. falciparum* Erythrocyte membrane protein 1 (EMP1) sequesters parasites in certain sites so that they are removed from the circulation and, consequently, do not travel to the spleen. This immunological effector organ efficiently filters PRBCs from the bloodstream (36). Although sequestration is advantageous for the survival of the parasite, this strategy has the pathological consequence of concentrating parasites in various vital organs. As sequestered parasites mature, they produce a variety of bioactive molecules that either upregulate or downregulate pathogenic processes. Immune responses to infectious insults are mainly initiated by the interaction of pathogen-associated molecular patterns (PAMPs) with receptors expressed by host cells. Many studies implicate glycosylphosphatidylinositol (GPI) of *P. falciparum* as a malaria PAMP and as a toxin (29).

It has long been recognized that factors endogenously produced by the host in response to parasite components contribute to the development of pathological conditions. Following his demonstration that malarial fever is synchronous with the developmental cycle of the blood-stage parasites, Camillo Golgi suggested already in 1886 that a toxin causes the malarial paroxysm. In 1899 Robert Koch also deduced the existence of a toxin and proposed the development of anti-toxic or clinical immunity (37). Maegraith proposed that both fever and other pathological processes in malaria were the result of a parasite toxin. He further exemplified the toxic basis of severe malaria by reference to the clinical similarities to bacterial sepsis, understood to be caused by endotoxin, and suggested that malaria toxin exerts similar effects through the induction of endogenous mediators of host origin (38).

P. falciparum GPIs elicit a wide range of activities, which resemble those produced during malaria infection. The observed in vitro effects of GPIs include: production of acute-phase cytokines such as TNF and IL-1; insulin-like activity, causing hypoglycemia and triglyceride lipogenesis in adipocytes; expression of inducible nitric oxide synthase in macrophages and endothelial cells; and upregulation of surface expression of intercellular-adhesion molecule 1, vascular cell-adhesion molecule 1 and E-selectin in leukocytes and endothelial cells (37, 39, 40). Consistent with these findings, specific antibodies could efficiently block the bioactivities of GPIs (41). GPI-induced cellular activation is mediated mainly by TLR2, initiating the MAPK and NF- κ B signaling pathways (40). These findings have been extended to other pathogens, e.g. *Trypanosoma brucei*, *Trypanosoma cruzi* and *Trypanosoma gondii*, confirming that GPIs act widely as pro-inflammatory toxins in eukaryotic parasitism (42). The culmination of these studies was the recent demonstration that murine antibodies raised through immunization with a *P. falciparum* GPI glycan analogue completely abolished parasite lysate-induced TNF- α release from mouse macrophages (43). To extend that the in vitro model matches the clinical situation in humans, this is the most

suggestive evidence to date that GPIs are the predominant pro-inflammatory toxins of *P. falciparum*. However, at present, the evidence that *P. falciparum* GPIs act as toxins in humans in vivo is circumstantial (44).

1.2.3 Structure, Recognition and Processing of *P. falciparum* GPIs

GPIs are a group of glycolipids that are expressed ubiquitously by eukaryotic cells (45). The primary role of GPIs is anchoring functionally important proteins onto cell surfaces, comprising molecules that provide barriers against immune attack and proteases, membrane property regulators, receptors, cell adhesion molecules, and cell surface hydrolases (46). In mammalian cells, GPI occurs in low abundance and is predominantly found in the protein-anchored form. In protozoan parasites, however, GPIs occur at relatively high levels both as moieties that anchor proteins and as glycolipids not attached to proteins (free GPIs). It is possible, although not yet proven, that it is the free GPIs released at the time of schizont rupture that are predominantly responsible for toxin-mediated effects. The glycan part of the GPI glycolipid consists of a conserved, linear 6-*O*-(ethanolamine-PO₄)- α -Man-(1-2)- α -Man-(1-6)- α -Man-(1-4)- α -GlcNH₂-(1-6)-*myo*-inositol-1-PO₄ core structure. GPIs from different organisms, species or cell types vary widely in the type of lipid moieties, inositol acylation, the extent of additional ethanolamine phosphate groups and sugar residues attached to the conserved glycan core (Fig. 2). These variations confer broad structural diversity and might impart a wide range of functional roles to GPIs.

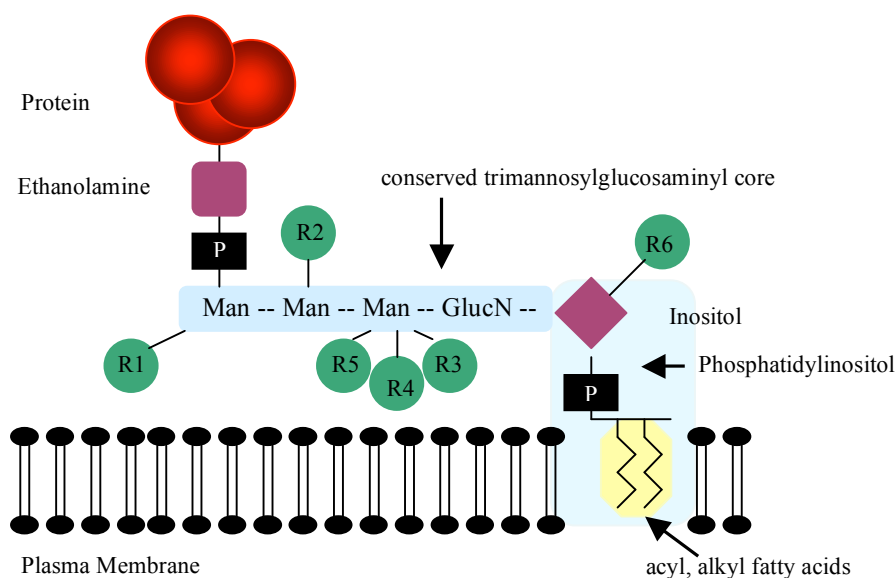


Figure 2. GPI-anchor structure and possible modifications to Man₃-GlucN oligosaccharide core. R1 = Man_α-(1-2); R2 = Phosphoethanolamine; R3 = Phosphoethanolamine; R4 = Gal₄; R5 = GalNAc_β-(1-4); R6 = Fatty acid at C₂ or C₃ of inositol.

The GPI anchor is linked with the carboxy-terminus of proteins via the ethanolamine head. Biosynthesis of GPI precursors and posttranslational protein modification with GPI proceed in the endoplasmatic reticulum. After post-translational removal of the carboxy-terminal signal peptide the resulting new terminal carboxyl group is combined with the amino group of the ethanolamine residue of a GPI precursor molecule. Then the GPI-anchored proteins are transported to the Golgi complex and are finally inserted into the plasma membrane (47).

P. falciparum synthesizes two mature GPIs at the mid to late trophozoite stages, which were shown to be Man4-GlcN-PI and Man3-GlcN-PI (48-49). The structures of *P. falciparum* GPIs are heterogeneous with respect to fatty acid substitution on the glycerol and inositol residues (Fig. 3). Plasmodial GPIs from human and rodent malaria species demonstrate a high level of conservation in structure, which is also seen in widely dispersed geographical isolates of *P. falciparum*. To date, the GPIs of the other human malaria parasites have not been described. The structures of parasite GPI differ considerably from those of human GPIs and therefore, *P. falciparum* GPIs comprise epitopes that can be recognized by the mammalian immune system and their biosynthetic pathway might offer a specific target for the development of novel drugs against malaria.

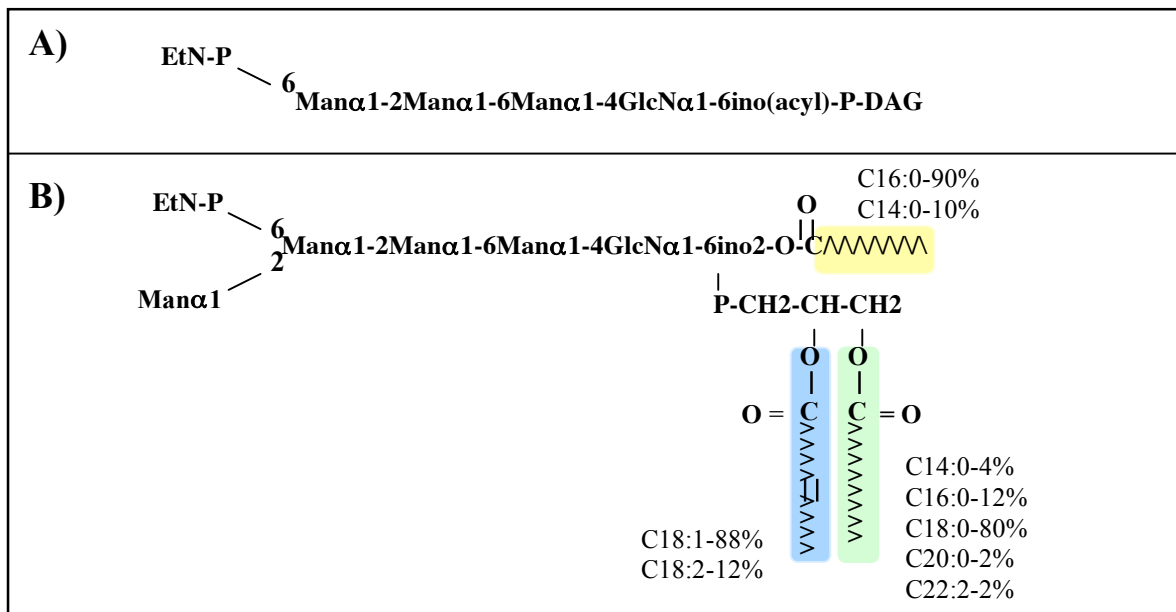


Figure 3. The structures of *P. falciparum* GPIs: A) Man3-GlcN-PI and B) Man4-GlcN-PI (46, 48).

Initiation of immune responses to *P. falciparum* GPIs likely occurs during schizogony, when merozoites with GPI-linked surface proteins and free GPI are released. GPIs may activate antigen presenting cells (APC) and macrophages by TLR2 (40). Parasite material is internalized by APCs and after processing through the endosomal/lysosomal pathway, peptides are presented in association with MHC class II to T cells. Given the absence of peptide epitopes for T cells, antibody responses to free GPIs are likely to be T-cell-independent during a primary infection. During a

secondary response, activated GPI-specific B cells may internalise and process GPI-linked proteins and present peptides derived from these GPI-linked proteins to CD4⁺ T cells in the context of MHC-II. It is probable that T-cell help for GPI-specific B cells is provided by T cells recognizing peptide epitopes that are physically linked to GPI, leading to immunoglobulin class switching, somatic mutation and affinity maturation of the BCR and the generation of plasma cells and memory B cells. Alternatively, GPIs could also be presented to NK T cells by CD1 molecules, which are MHC-like molecules expressed on the surface of APCs (44).

1.2.4 GPI-anchored proteins of *P. falciparum*

In addition to their putative roles as toxins, GPIs may indirectly contribute to malaria pathogenesis by anchoring protein determinants of parasite virulence. The surfaces of the various extra-cellular forms of the malaria parasite, the merozoite, gamete, ookinete, and sporozoite, are coated by different proteins that are either known or presumed to be GPI-anchored. Some of these proteins are well characterised including MSP-1 and MSP-2 on merozoites (50, 51), Pfs48/45 on gametes (52), Pfs25 and Pfs28 on ookinetes (53, 54), and CSP on sporozoites (55, 56). Most known blood-stage GPI-anchored proteins appear to be essential for growth as attempts to generate parasite “knock-out” mutants have failed (57) and several of them are prominent vaccine candidate antigens.

Genomic and proteomic studies suggest that only a minority of GPI-anchored surface proteins of *P. falciparum* has been discovered so far (58-60). The C-terminal sequences of known GPI-anchored proteins share the general characteristics of eukaryotic GPI anchor signals, namely a C-terminal stretch of 13-18 non-charged amino acids enriched with hydrophobic amino acids. The cleavage sites of three *P. falciparum* GPI anchor signal sequences are known (MSP-1, -2, and -4) (61). Despite this knowledge, very few *P. falciparum* proteins have been confirmed as possessing GPI anchors. Due to fatty acid acylation of inositol in the glycolipid structure, GPI-anchored *Plasmodium* proteins are resistant to cleavage by phosphatidylinositol-specific phospholipase C, thereby preventing the use of this common enzymatic identification method (48). To identify more GPI-anchored proteins of *P. falciparum* a combination of proteomic and computational approaches has been used. Focusing on the blood stages, proteomic analysis of proteins labelled with radioactive glucosamine identified GPI anchoring on 11 proteins ((MSP)-1, -2, -4, -5, -10, rhoptry-associated membrane antigen, apical sushi protein, Pf92, Pf38, Pf12, and Pf34). Another nine lower abundance [³H]-glucosamine-labelled GPI-anchored proteins were also apparent at this life stage, but these remained unidentified. To predict the identity of these proteins as well as those present at other life stages, bioinformatic approaches were developed and predicted that the *P. falciparum*

genome encodes 30 GPI-anchored proteins (62). Since sporozoites cannot be cultivated *in vitro*, this approach cannot be applied for this development stage.

Analysis of the GPI-anchored proteome of different life stages of *P. falciparum* will form an important basis for further elucidation of the complex host-pathogen interactions in malaria. Characterisation of newly identified GPI-anchored proteins will contribute both to the understanding of host cell invasion mechanisms and the nature of anti-parasite immunity.

1.2.5 Immunity to *P. falciparum* GPIs

Epidemiological studies show that, after the initial period in which children are susceptible to severe malaria, protective immunity to malaria develops in three sequential phases: first, immunity to life-threatening disease which operates despite high prevalence of parasitaemias; second, immunity to symptomatic infection which also occurs in the face of relatively high prevalence, incidence and density of infection; and only then, third, partial immunity to parasitization that limits parasite numbers, replication and burden within the host (29). Natural immunity to malaria is therefore thought to be a combination of anti-toxic and anti-parasitic immune responses. It appears that anti-toxic immunity, or anti-disease immunity may be distinct processes from anti-parasite immunity (63). The former are acquired both earlier and more easily. Anti-parasite immunity is difficult to achieve and unstable, meaning that ongoing exposure is required to maintain immunity (37).

One possibility is that anti-toxic immunity results in part from the acquisition of acquired immune responses to GPI. Young children are compromised in their ability to mount anti-carbohydrate responses and this might contribute to their susceptibility to severe disease. In populations exposed to *P. falciparum* the antibody response to purified GPIs is characterised by a predominance of IgG over IgM and an increase in the prevalence, level and persistence of responses with increasing age (44). This has raised the possibility that these naturally occurring antibodies could neutralize GPIs and influence the outcome of human malaria. The relationship between anti-GPI antibodies and clinical malaria has been evaluated in a number of studies. In children in Western Kenya a significant association was found between the presence of anti-GPI antibodies and reduced risk of severe anaemia. In contrast, people who were not exposed to the malaria parasite completely lacked anti GPI-antibodies (61). Subsequent cross-sectional studies reported no association between presence of anti-GPI antibodies and protection against mild clinical malaria (64), severe malaria (65) or maternal immunity (66); similarly, a prospective study also found no association between anti-GPI antibodies and resistance to clinical malaria (65). Conversely, in a recent study from Senegal, anti-GPI antibody concentrations were significantly lower in cerebral

malaria patients than in those with mild malaria (67), and anti-GPI antibodies were associated with markedly reduced risk of symptomatic infection in children of Javanese migrants to Papua (68).

One plausible explanation for the variable results of epidemiological studies resides in the fact that GPIs are not readily amplifiable from natural sources, and their purification often yields heterogeneous material. In addition, some of the protocols used to measure anti-GPI antibody levels may be suboptimal: the extracted GPI material was immobilized non-covalently via unspecific interactions to ELISA plates and subsequently washed with detergent-containing buffer. These conditions favor an uncontrolled loss of material with resulting loss of sensitivity and specificity of the assay. To date, however, there is little evidence to show that the antibody responses measured correlate with protection against clinical malaria or disease severity and clearly, this area requires considerable further investigation. The use of fully synthetic GPIs will help dispel any doubt that the responses measured are specific, and combining clinical studies with functional assays (such as toxin neutralisation) will provide the clearest outcomes (44).

1.2.6 Prospects for Anti-GPI Vaccination

Vaccination is an elegant way to induce an adaptive immune response in non-immune individuals by confronting the immune system with crucial antigenic determinants of a pathogen without the deleterious effects of disease. Despite decades of research, an effective vaccine against malaria has remained elusive. There are, however, several lines of evidence to suggest that protective immunity to malaria is possible. Repeated infection of an individual with malaria leads to the gradual acquisition of naturally acquired immunity (sect. 1.2.5). Immunization with irradiated sporozoites has been shown to result in partial or complete protection in mice (69), monkeys (70) and humans (71) from experimental malaria challenge with viable sporozoites. Further, passive transfer of purified immunoglobulins from immune individuals can protect children with acute infection (72). However, key obstacles to the development of a malaria vaccine include the lack of immune correlates of protection, the lack of reliable and predictive animal models, and the developmental and antigenic diversity and variability of the parasite.

Nevertheless, development of a vaccine against malaria that reduces morbidity and mortality has long been seen as an essential component of a sustainable malaria control strategy. The majority of subunit-vaccine candidates, whether against sporozoites, liver-stages, gametocytes, merozoites or trophozoite blood-stages, target proteins expressed either on the surface or within invasion organelles of these life-stage, and would provide clinical protection indirectly, by killing the parasite or by reducing replication and hence parasite burden. All these strategies face similar problems, e.g. redundancy in invasion pathways, antigenic diversity, antigenic variation,

immunosuppression, immune evasion strategies and problems of MHC-linked genetic restriction in the immune response to these antigens (37). A multivalent multi-stage approach may thus be required.

The use of vaccines against the disease aims to reduce morbidity and mortality directly, by immunizing individuals with parasite products that contribute to host pathology. In this case, the death of the parasite is not a necessary objective in fulfilling of the aims of the vaccine (37). Chemical synthesis of the glycan group of *P. falciparum* GPI has recently provided a means to test the hypothesis that this molecule is causally involved in the pathogenesis of *P. berghei* infection. Mice immunized against the GPI glycan were substantially protected against severe malarial pathology, including cerebral syndrome, acidosis and pulmonary oedema, and fatality. The noted protection was independent of any reduction in parasitaemia (43). These findings indicate that GPI is an essential parasite product in the pathogenesis of systemic disease and the lethal syndromes, at least in this model. GPI may therefore also contribute to pathogenesis and fatalities in humans. This data provide pre-clinical proof-of-principle for the development of an anti-toxic vaccine against malaria. However, vaccination targeted at GPIs in humans raises safety concerns that vaccinated individuals with malaria would feel less sick and thus present to hospital later and with high levels of parasitaemia, with the attendant risks of severe anaemia and organ failure. Inhibition of GPI-mediated early pro-inflammatory cytokine responses that limit parasite growth may also favour rapid raises in parasitaemia in vaccinated individuals (73). Thus, although the preclinical data indicate a role for GPI in severe pathogenesis, it still remains to be determined whether anti-toxic vaccines can be deployed alone (where they may prevent or delay severe pathology) or whether the vaccine needs to be given in combination with an effective anti-parasite vaccine (in order to prevent the possibility that, if acute clinical symptoms are effectively suppressed, cases may take longer to present for treatment) (74).

1.3 Anthrose for Anthrax Detection

1.3.1 *Bacillus anthracis* and Anthrax

The fifth and sixth plagues in the Bible's book of Exodus may have been outbreaks of anthrax, caused by *Bacillus anthracis*. *B. anthracis* is derived from the Greek word for coal, anthrakis, because of the black skin lesions it causes. This obligate pathogen exists in two forms, the vegetative form and the spore. The vegetative form is a facultatively anaerobic, large, Gram-positive bacillus, which, under adverse growth conditions, forms a single endospore. The spores are remarkably resistant to a range of adverse environmental conditions, e.g. temperature, desiccation, pH, chemicals, or irradiation, thus ensuring long-term survival (75). Anthrax is a zoonosis whose

natural reservoir may be considered to be the soil. There is no animal reservoir, because infected animals rapidly die. Anthrax is a disease largely of mammalian herbivores, which acquire their infection through ingestion of contaminated soils. Secondary spread may occur directly to scavenger animals, via flies contaminating vegetation, or via biting flies (Fig 4). If spores are ingested, disease begins in the intestinal tract, with subsequent development of septicemia. In the case of scavenger animals that eat dead animals, the disease results from infection by vegetative organisms. Illness in animals progresses rapidly, and often the first sign of anthrax is the discovery of dead animals (76).

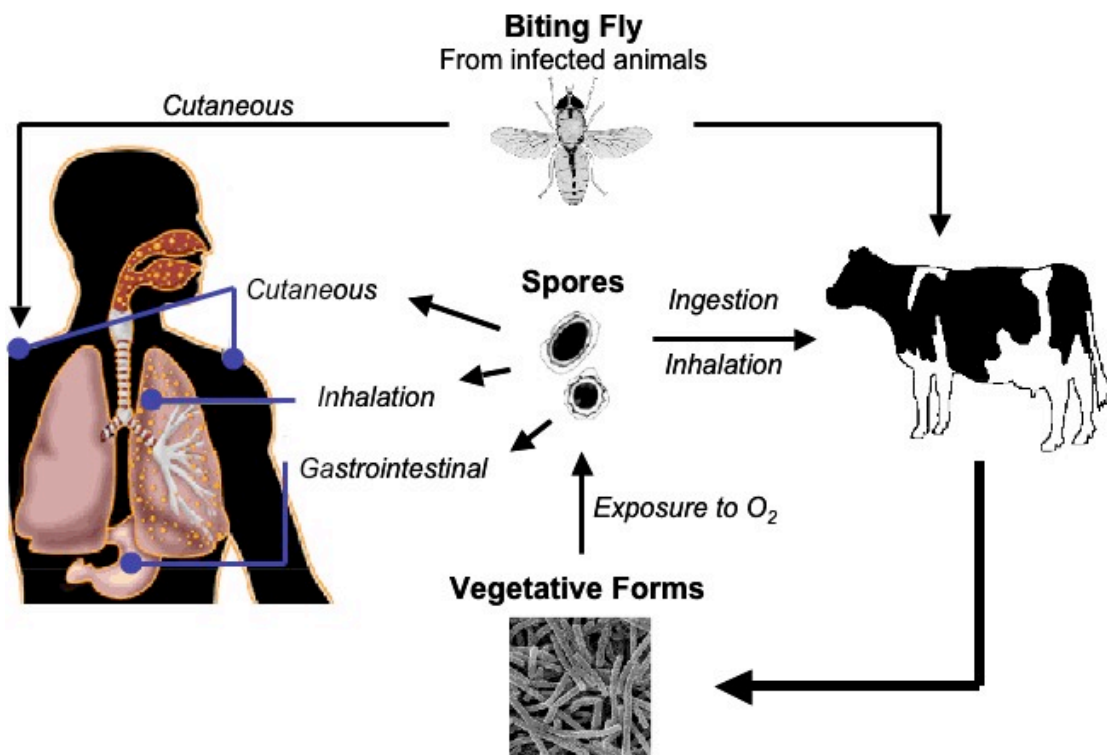


Figure 4. Cycle of infection in anthrax. The spore is central to the cycle, although infection can also be acquired through uptake of the vegetative forms when humans or carnivores eat meat from an animal that died of anthrax or when biting flies transmit the disease (75).

Outbreaks in animal populations are prevalent and widespread. An outbreak in Iran caused the death of 1 million sheep in 1945; an epidemic in South Africa and Namibia between 1984 and 1989 killed thousands of wild animals; 19 outbreaks in 1996 in Nepal affected 222 animals; 1,570 cases of anthrax in cattle in China were reported in 1996; the death of 204 livestock in Australia was reported in 1997; and 1,612 outbreaks occurred between 1991 and 1996 in India. Cattle and sheep are the usual victims and have a mortality rate of 80%, though horses and swine are also affected. Wild animals, including deer, many rodents, toads, and cat species are at risk, while frogs, rats and adult dogs are largely resistant (77, 78).

Once in the host, spores are phagocytized by macrophages and are carried to the lymph nodes, during which time they germinate and multiply. As the capacity of the lymph nodes is exceeded, the bacteria then enter the blood stream and eventually cause septicemia. The principal virulence factors of *B. anthracis* are its capsule and two exotoxins, all of which are coded on one of two plasmids, the pX01 and pX02 plasmids. Both plasmids are necessary for full virulence; loss of either results in an attenuated strain. Sterne, such an attenuated strain, carries pX01 and therefore can synthesise exotoxin, but does not have a capsule. The poly-D-glutamic acid capsule inhibits phagocytosis of vegetative cells and is also poorly recognized by the host's immune system. The anthrax toxin is composed of three proteins known as protective antigen (PA), lethal factor (LF), and edema factor (EF). These proteins must act cooperatively, with PA containing the cell-binding domain, and either LF or EF causing cell toxicity. PA binds to receptors on the surface of most cell types, though macrophages are typically most affected. PA is then activated by proteolytic cleavage which leaves a 63-kDa fragment (PA63) attached to the receptor. The loss of the 20-kDa fragment (PA20) triggers heptamer formation by receptor-bound PA63 and exposes a hydrophobic patch on each monomer of PA63, which can then bind to either EF or LF in a competitive manner. The formation of the PA63 heptamer induces endocytosis and concurrent transport of EF or LF into the cell. Edema toxin produces tissue edema, inhibits neutrophil activity, and interferes with production of TNF and IL-6 by monocytes. Lethal toxin interferes with intracellular signaling by inactivating mitogen-activated protein kinase. Lethal toxin also stimulates the release of TNF and Il-1 from macrophages, which may contribute to death in anthrax toxemia (78-80).

1.3.2 Anthrax in Humans

The major sources of human anthrax infection are direct or indirect contact with infected animals, or occupational exposure to infected or contaminated animal products. Accidental human infection by *B. anthracis* is rare; in the 1980s, there were only 2.000 estimated annual worldwide cases (77, 78). It is believed that approximately 80% of reported cases of anthrax infection were due to industrial exposure, with the majority of the remaining cases being caused by agricultural exposure. Widespread use of vaccines in high risk populations of animals and humans, as well as improvements in industrial hygiene have minimized such occurrences in industrialized nations (78, 81, 82).

Anthrax is classified as a disease by the route of exposure, as exposure to spores via cutaneous, inhalation, or oral ingestion has different pathological consequences. Cutaneous anthrax is the most common type, with an estimated 2.000 cases reported annually worldwide. The largest epidemic occurred in Zimbabwe in 1979-1985, when more than 10.000 human cases were reported (83). Cutaneous anthrax occurs at the site of inoculation of spores into the skin via an abrasion, cut

or possible insect bite. Cutaneous anthrax causes mortality in approximately 20% of cases if not treated, but is entirely treatable with appropriate antibiotics. Cutaneous anthrax begins as a pruritic papule that becomes vesicular, and then necrotic, often accompanied by significant edema, consistent with the expression of the toxin at the site of infection. A characteristic painless black edematous lesion develops, and then resolves in 1 to 2 weeks. Most cases of cutaneous anthrax resolve spontaneously. The most deadly form of anthrax is pulmonary anthrax, or inhalational anthrax, which results from the inhalation of airborne spores (78). Inhalational anthrax develops slowly over a period of 1–6 days and causes general malaise, coughing, and fatigue followed by more serious respiratory distress. Death is reported to occur within 24–36 h after respiratory distress begins (78, 79). Even with early intervention, the prognosis of patients with inhalational anthrax is poor: inhalational anthrax has a mortality rate nearing 100%. Gastrointestinal anthrax is the rarest form of natural anthrax and outbreaks are continually reported mostly in Africa and Asia, following ingestion of insufficiently cooked contaminated meat. Gastrointestinal anthrax causes initial gastroenteritis with progressively worsening abdominal complications including severe diarrhoea, abdominal pain, and vomiting of blood. Late-stage gastrointestinal anthrax leads to bowel perforation and toxemia resulting in death within 2–5 days. Gastrointestinal anthrax has been reported to cause fatalities in 25–60% of cases. Fortunately, *B. anthracis* is sensitive to a variety of antimicrobials. The effectiveness of treatment depends on the route of exposure and the time of diagnosis. Antibiotic treatment is highly effective in cases of cutaneous anthrax resulting in sterilization of the lesions within 24 hours and resolution within a few weeks. Typical antibiotics include ciprofloxacin, doxycycline, and penicillin. However, once the infection is established, successful medical intervention is difficult as the infection becomes refractory to treatment (78).

Although anthrax is susceptible to antibiotics, vaccination would be the most efficient and cost effective form of mass protection. An attenuated *B. anthracis* strain, isolated by L. Pasteur in 1881, was used successfully in animals as the first live bacterial vaccine (84, 85). The Sterne attenuated spore vaccine, based on an avirulent non-encapsulated 34F2 strain is still used as a live veterinary vaccine (86). In contrast to the former USSR and China, live spore vaccines have not been adopted for human use in Western countries due to fears concerning residual pathogenicity (86, 87). The current U. S. vaccine for anthrax has been licensed since 1972 and was developed based on the outcome of human trials conducted in the 1950s (88). This vaccine, known as anthrax vaccine adsorbed (AVA), consists of a culture filtrate from an attenuated strain of *B. anthracis* (V770-NP1-R) adsorbed to aluminum salts as an adjuvant. The principal antigenic component of AVA is PA antigen, with smaller amounts of EF and LF. A similar culture supernatant-derived human vaccine is produced in the United Kingdom. The UK vaccine is from the animal vaccine Sterne strain (89). The vaccine is licensed to be given in a six dose series over an 18-month period.

In a study, using a monkey model, inoculation with this vaccine at zero and two weeks was completely protective against an aerosol challenge at eight and 38 weeks and 88% effective at 100 weeks (90). Although the current vaccines available provide effective protection, they do suffer from several problems, namely: lack of standardisation, the relatively high expense of production, the requirement for repeated dosing, and the associated transient side effects (91). Much of the work on generating a second generation vaccine is based on the observation that antibodies to PA are crucial in the protection against exposure to virulent anthrax spores. Antibodies to PA are thought to prevent binding to its cellular receptor and subsequent binding of LF and EF, which are required events for the action of the two toxins. Protective immunity in key animal models can be generated by vaccination with recombinant PA, as well as spores and DNA plasmids that express PA. Although antibodies to PA address the toxemia component of anthrax disease, antibodies to additional virulence factors, including the capsule or somatic antigens in the spore, may be critical in development of complete, sterilizing immunity to anthrax exposure (92).

1.3.3 Anthrax as Biological Weapon: Need for Rapid-Detection Methods

Bioterrorism is defined by the intentional or threatened use of microorganisms or toxins derived from living organisms to cause death or disease in humans, animals or plants on which we depend. The other major point is to generate fear in the population (93). Many such agents are zoonotic and could have a considerable impact on agriculture as well as on human health. Anthrax is seen as one of the most likely biological agents for use as a weapon. *B. anthracis* spores are quite stable in the environment, can be transmitted by aerosolisation and cause inhalational anthrax that has a high mortality rate. It has been estimated that 50 kg of *B. anthracis* spores released over an urban population of 5 million would sicken 250.000 and kill 100.000 (94). This would almost certainly lead to a rapid total breakdown in medical resources and civilian infrastructures.

Its applicability for use in biological warfare has been proven throughout the 20th century. During World War I, Germany used *B. anthracis* to infect livestock during transshipment. In 1941 the British Government began testing the effect of anthrax on sheep on the Scottish island of Gruinard. Due to the durability of the spores the island was declared safe only in 1990 after decontamination using formaldehyde (83). Japan infected prisoners and contaminated the waterways and food supplies of various Chinese cities with *B. anthracis* between 1932 and 1945 (95). The United States has weaponised anthrax spores, as did other countries in the 1950s and 1960s. This was evidenced by the accidental aerosol release of *B. anthracis* spores from a Soviet military microbiology facility in Sverdlovsk in the former Soviet Union in April 1979. This was the largest known outbreak of inhalational anthrax in the 20th century: 68 of the 79 patients with

inhalational anthrax died (96). In more recent times, *B. anthracis* has also become a threat from terrorist groups. The cult of Aum Shinrikyo in Japan tried on several occasions to disperse anthrax unsuccessfully in Tokyo in the mid 1990s, but they used an attenuated vaccine strain (83). The most recent biological warfare attack was the deliberate release of *B. anthracis* in the United States shortly after the terrorist attacks of 11 September 2001. This terrorist act caused 22 cases of anthrax, including 5 deaths. To prevent future attacks *B. anthracis* biology and pathogenesis has been studied in search for improved vaccines and detection methods (93).

Due to the potential of *B. anthracis* for use as an agent of bioterrorism, its occupational exposure, and the persistence of spores in the environment, the development of rapid and accurate detection methods are needed. Aside from these exposure factors, rapid diagnosis of anthrax is necessary since the infection is difficult to diagnose (78). Specific detection of *B. anthracis* is challenging, due to its genetic similarity to other bacteria of the *B. cereus* group, which comprises *B. cereus*, *B. anthracis*, *B. thuringiensis*, and *B. mycooides* (97). Genomic studies on strains of *B. anthracis*, *B. cereus* and *B. thuringiensis* have suggested that *B. anthracis* and *B. thuringiensis* are sub-species of the species *B. cereus*. The main difference between these sub-species is the presence of plasmids coding for insecticidal toxins in *B. thuringiensis* and the presence of the capsule and toxin plasmids in *B. anthracis* (78). Although it is not possible to discriminate between species by 16S rRNA sequencing, *B. anthracis* can be clearly distinguished using a range of DNA-based approaches such as amplified fragment length polymorphism, variable number tandem repeat (VNTR) and multilocus sequence typing (MLST) (98, 99). In addition, although *B. anthracis* is one of the most molecular monomorphic bacteria known, all known strains have been separated into five categories (allowing for geographical identification) on the basis of variable numbers of tandem repeats in the variable region of the *VrrA* gene (100). Individual isolates can now be typed on the basis of single-nucleotide polymorphisms (101). These PCR-based assays and traditional phenotyping of bacteria are the most accurate detection systems but are also complex, expensive, and slow. Therefore, there is a pressing need to develop novel methods for rapid, simple, and precise detection of *B. anthracis*. Currently, anthrax detection research is mainly based on nucleic acid detection, immunoassays and mass spectrometry.

1.3.4 Unique Anthrose carried by the Exosporium Glycoprotein BclA

All members of the *Bacillus* genus produce endospores as part of their life cycle. It is not possible to determine the identity of spores by casual or morphological examination and therefore, identification and classification of spore species have focused on the vegetative form of these organisms (sect. 1.3.3). The mature *B. anthracis* spore contains a central, genome-containing

compartment called the core and three prominent protective layers called the cortex, coat, and exosporium (102). The cortex, which is adjacent to the core, is composed of a thick layer of peptidoglycan (103). The cortex is covered by the spore coat, which contains approximately 30 different proteins (104). The exosporium consists of a paracrystalline basal layer and an external hair-like nap. As the outermost layer, the exosporium serves as the primary permeability barrier of the spore and as the source of spore surface antigens (102, 105). In addition, the exosporium interacts with the soil environment, with spore-binding cells in the mammalian host, and with host defenses. The formation of the exosporium during sporulation is a complex and energy-consuming process. As such, it is unlikely that this structure would be maintained by the bacterium unless it was important for survival. The pressure to maintain the exosporium is probably related to some aspect of infection because most non-pathogenic *Bacillus* spores do not possess a well-developed exosporium (106).

The glycoprotein *Bacillus* collagen-like protein of *anthracis* (BclA) is the structural component of the hair-like nap and has been shown to be an immunodominant protein suggesting a role for this protein in spore-host interactions (105). BclA has conserved amino- and carboxy-termini and a long, central collagen-like region that is similar to mammalian collagen proteins (107, 108). This polymorphic collagen-like region consists of GXX repeats, including a large proportion of GPT triplets, and (GPT)₅GDTGTT repeats. The latter 21-amino-acid repeat has been named BclA repeat (107) and appears to be an essential feature of the BclA protein important for the structural organization of BclA on the spore surface. The number of GXX repeats in the collagen-like region found in different naturally occurring *B. anthracis* strains varies from 17 to 91 (105, 107, 108). The length of this region is proportional to the length of the hair-like nap, which varies from 140 to 608 Å (108). The *bcla* gene is also present in the genome of *B. cereus* and *B. thuringiensis* (109).

The BclA protein is extensively glycosylated with two *O*-linked carbohydrates, a 715-Da tetrasaccharide and a 324-Da disaccharide (110). Multiple copies of the tetrasaccharide are linked to the collagen-like region of BclA, whereas the disaccharide may be attached outside of this region. Most of the collagen-like region repeating units contain a threonine residue that provides sites for potential glycosylation via a GalNAc linker. It was assumed that BclA glycosylation plays an important role, since glycosylation requires many genes and enzymes and it occurs when the cell is starved for nutrients. For other prokaryotic glycoproteins, several different functions for glycosylation have been proposed, including the maintenance of protein conformation, heat stability, surface recognition, resistance to proteolysis, enzymatic activity, cell adhesion, agglutination, and immune evasion (110). Using NMR, mass spectrometry, and other analytical techniques, the structure of the tetrasaccharide was recently elucidated as 2-*O*-methyl-4-(3-

hydroxy-3-methylbutamido)-4,6-dideoxy- β -D-glucopyranosyl-(1 \rightarrow 3)- α -L-rhamnopyranosyl-(1 \rightarrow 3)- α -L-rhamnopyranosyl-(1 \rightarrow 2)-L-rhamnopyranose. A unique characteristic of this glycan is the previously undescribed terminal sugar (i.e. 2-O-methyl-4-(3-hydroxy-3-methylbutamido)-4,6-dideoxy-D-glucose), the so called anthrose. Since anthrose was not found in spores of the *B. cereus* T strain and a *B. thuringiensis* ssp. *kurstaki* strain, anthrose may be useful for species-specific detection of *B. anthracis* spores or as a new target for therapeutic intervention (110).

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CHAPTER 2: GOAL AND OBJECTIVES

2.1 Goal

To contribute to the development and evaluation of synthetically produced carbohydrate antigens of infectious agents as vaccine components and tools in biomedical research.

2.2 Objectives

1. To develop a novel carbohydrate microarray based on synthetic *Plasmodium falciparum* GPI glycans
2. To assess levels and fine specificities of anti-GPI antibodies in healthy and malaria exposed cohorts with the carbohydrate microarray
3. To select GPI specific B-cell hybridomas with the carbohydrate microarray
4. To evaluate biological activities of the produced monoclonal anti-GPI antibodies
5. To use synthetically produced *Bacillus anthracis* spore glycan antigens to produce monoclonal antibodies that allow spore detection

CHAPTER 3: SYNTHETIC GPI ARRAY TO STUDY ANTITOXIC MALARIA RESPONSE

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“A carbohydrate microarray provides insights into the antimalarial immune response. Kamena et al. (p 238) spotted synthetic GPI glycans onto glass slides to create a 'GPI chip'. Using this chip, the authors characterized the binding of anti-GPI antibodies from people in malaria-endemic and malaria-free regions and before and after malarial exposure (see also News and Views by Ferguson on p 223). Shown are images of the carbohydrate microarrays incubated with human sera, Giemsa-stained red blood cells infected with Plasmodium falciparum and the chemical structures of GPI glycans displayed on the microarrays. Cover art by Erin Boyle based on images provided by Faustin Kamena and Marco Tamborrini.” Nat Chem Biol, about the cover, April 2008 Vol 4 No 4.

SYNTHETIC GPI ARRAY TO STUDY ANTITOXIC MALARIA RESPONSE

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Abstract

Parasite glycosylphosphatidylinositol (GPI) is an important toxin in malaria disease, and people living in malaria-endemic regions often produce high levels of anti-GPI antibodies. The natural anti-GPI antibody response needs to be understood to aid the design of an efficient carbohydrate-based antitoxin vaccine. We present a versatile approach based on a synthetic GPI glycan array to correlate anti-GPI antibody levels and protection from severe malaria.

Malaria kills more than two million humans every year, most of them African infants under the age of two. While children living in malaria-endemic regions seem to develop immunity to severe malaria after only a few infections¹, immunity to infection is acquired much later². Natural immunity is therefore thought to be a combination of antitoxic and antiparasitic immune responses, and lack of antitoxic immunity may represent a key factor responsible for the vulnerability of infants to severe malaria. GPI has emerged as a central toxin that induces the expression of many host genes implicated in malaria pathogenesis². As for other parasitic diseases, such as trypanosomiasis and toxoplasmosis, certain pathogenic features may be caused by parasite GPI glycans^{3,4}.

GPIs anchor proteins in the membranes of all eukaryotic cells⁵ and constitute up to 90% of protein glycosylation in protozoan parasites⁶. A considerable fraction of GPI appears at the plasma membrane without protein attachment as free GPI (ref. 7). The glycan part of the GPI glycolipid consists of a conserved, linear 6-*O*-(ethanolamine-PO₄)-Man- α (1-2)-Man- α (1-6)-Man- α (1-4)-GlcNH₂- α (1-6)-*myo*-inositol-1-PO₄ core structure. Serological studies using GPIs purified from *Plasmodium falciparum* have indicated that sera from malaria-endemic regions contain naturally occurring anti-GPI antibodies^{5,8,9,10}. However, it is not clear to what extent the development of anti-GPI immune responses is related to malaria exposure, and there is a growing interest to assess the relevance of the anti-GPI immune response for protection against malaria disease. A recent study with a rodent malaria model showed that a GPI-based vaccine candidate substantially reduces malaria mortality¹¹. However, evidence for a correlation of anti-GPI antibody levels and antitoxic immunity against severe malaria in humans is missing. One explanation for this lack of data resides in the fact that GPIs are not readily amplifiable from natural sources, and their purification often yields heterogeneous material. Several studies using bulk-purified GPIs from cultured parasites in ELISA experiments have yielded contradictory results^{5,8,9,10}. Moreover, some technical aspects of those studies remain questionable. GPIs are amphiphilic molecules and therefore are poorly soluble in pure alcohol. However, lyophilized GPI-containing fractions from the studies mentioned above^{5,8,9,10} were resolubilized in pure methanol. In addition, the GPI material was immobilized noncovalently via unspecific interactions to ELISA plates and subsequently washed with detergent-containing buffer. These conditions favor an uncontrolled loss of material and might in part explain the inconsistency of the results obtained. Here, we present a versatile method based on microarrays of covalently attached synthetic GPI glycans for parallel screening of multiple sera on a single slide.

We developed a high-throughput screening platform by spotting picomolar amounts of synthetic GPI glycan fragments of different lengths on the surface of glass slides (Fig. 1). Each synthetic molecule was spotted in quadruplicate in order to ensure reproducibility of the results. In addition, the molecules were arrayed to create 64 identical screening units on a single glass slide.

The printed glass slides were then assembled to a 64-well screening platform with an adhesive gasket on the surface of the slide (Supplementary Methods and Supplementary Fig. 1 online). The amount of synthetic molecule immobilized (5–10 pg) and the volume of the test sample (5 μ l) make this platform an excellent tool for screening large numbers of serum samples. To validate the printing of carbohydrates on the microarray, we immobilized decreasing concentrations of oligosaccharide **6** on microarray slides and incubated the slides with fluorescein isothiocyanate (FITC)-labeled ConA. We observed a linear correlation between the concentration of GPI **6** and binding to ConA. This confirms the direct relation between the concentration of the sample and the amount of immobilized carbohydrate (Supplementary Methods and Supplementary Fig. 2 online).

Analysis of synthetic *P. falciparum*-related GPI glycans of different lengths revealed a length-dependent binding of the GPI glycan to paired serum samples collected from healthy subjects in a malaria-endemic region in both wet and dry seasons (Fig. 2a–f). Glycan fragments containing less than five carbohydrate units (**1**, **2**, **3**) showed virtually no binding to the serum samples tested, whereas longer glycans (**4**, **5**, **6**, **7**) all showed substantial bindings (Fig. 2a–f). This result suggests that a pentasaccharide likely represents the minimal epitope for naturally elicited anti-GPI antibodies.

Sera taken from individual subjects living in the same malaria-endemic area diverged substantially in the fine specificity of anti-GPI antibodies, as illustrated by some examples (Fig. 2a–f). Though some sera contained antibodies that only recognized the GPIs with a phosphate ethanolamine group on the third mannose (Fig. 2b,e), most other sera reacted with all Man₃- and Man₄-GPIs (**4**, **5**, **6**, **7**). Paired samples taken in a 6-month interval from the same donors revealed a notable stability of individual anti-GPI antibody response patterns, irrespective of overall antibody level increase (Fig. 2a–e) or decrease (Fig. 2e,f). To validate the microarray method, we performed comparative serum titration experiments in ELISA versus microarray-based analysis with 12 selected sera and found good correspondence of results (Supplementary Methods and Supplementary Fig. 3 online).

In a more detailed analysis, we assessed the antibody levels of serum samples collected from malaria-exposed and non-exposed populations to the two structurally distinct GPI glycans with or without the ethanolamine phosphate group (Man₄-GPIs **6** and **7**, and Man₃-GPIs **4** and **5**) of *P. falciparum*. Sera from malaria-exposed adult Africans had high IgG levels against Man₄-GPIs **6** and **7**, and substantial reactivity was observed against the Man₃-GPIs **4** and **5**, although the mean antibody level was lower than that observed against the Man₄-GPIs (Fig. 2g). Thus, the fourth mannose was identified as a key recognition element for many GPI-reactive antibodies. The phosphate ethanolamine group on the third mannose generally does not have an important role in

binding. Mean antibody levels against GPI glycans **4** and **6**, which lack this appendage, were only slightly lower than those against compounds **5** and **7**, respectively.

Surprisingly, most sera of malaria-unexposed Caucasian adults also contained IgG that bind to the Man₄-GPIs **6** and **7** (Fig. 2h). In contrast to sera from malaria-exposed Africans, no significant reactivity with the Man₃-GPIs **4** and **5** was observed (Fig. 2h). Both Man₃-GPI **5** and Man₄-GPI **7** have been found on *P. falciparum* blood-stage parasites^{12,13}. The ratio of the two structurally distinct molecules may vary during intra-erythrocytic development¹⁴. Only Man₄-GPI serves as anchor for surface proteins such as MSP-1 and MSP-2 (ref. 13). *P. falciparum* Man₃-GPI, like some *Toxoplasma gondii* GPIs, may primarily exist as the free glycolipid on the parasite cell surface. Screening with microarrays of structurally defined synthetic GPIs demonstrates that both structures comprise epitopes that can be recognized by the human immune system. This finding indicates that the Man₄-GPI response represents a broader anti-GPI response probably also involving some nonmalarial species, whereas the Man₃-GPI response might represent a more specific antimalarial GPI response.

The difference in the anti-Man₃-GPI response between African and European sera may represent a response to nonmalarial parasites found only in Africa. To address this point, we hypothesized that if anti-Man₃-GPI responses are primarily elicited by malaria parasites, whereas anti-Man₄-GPI antibodies arise from exposure to a variety of pathogens, a malaria infection in previously unexposed individuals should boost a pre-existing anti-Man₄-GPI B-cell response and should also elicit a de novo anti-Man₃-GPI response. In support of this hypothesis we observed an increase in pre-existing anti-Man₄-GPI (**6** and **7**) IgG levels and the development of a substantial anti-Man₃-GPI (**4** and **5**) antibody response in Caucasian volunteers involved in a sporozoite challenge vaccine trial (Fig. 2i). This finding is even more noteworthy when considering that antimalaria chemotherapy started immediately after the first microscopic detection of blood-stage parasites. These results demonstrate that even one subclinical malaria infection can have a profound effect on the overall anti-GPI antibody level and on the reactivity pattern.

Our approach provides more detailed insights than those obtained with antigens extracted from parasite preparations. Anti-GPI fine specificity patterns likely reflect exposure to a variety of pathogens and mirror the general epidemiological situation of a population. Therefore, anti-GPI antibody levels measured with GPI isolated from *P. falciparum* parasites critically depend on the ratio of Man₃- and Man₄-GPI in the antigen preparations. The ratio of mean anti-Man₃-GPI and anti-Man₄-GPI antibody levels may represent a marker for the relative intensity of malaria exposure.

The immune system of children under two years of age is unable to produce antibodies to many carbohydrate antigens unless the sugars are conjugated to a carrier protein¹⁵. Lack of antibodies to GPI in small children may stem from the fact that a free glycolipid (rather than the protein-GPI conjugates) is predominantly responsible for induction of anti-GPI antibodies⁹. We observed that a specific response against the Man₃-GPI, presumably the free form of GPI in *P. falciparum*, was built up after host contact with the malaria parasite. The observation that European control subjects also showed a substantial anti-Man₄ response poses an important question, as the origin of that response could be of major importance for the design of a GPI-based antitoxin malaria vaccine. A possible explanation is that the anti-Man₄-GPI response in malaria-unexposed subjects may arise as a result of an infection with nonmalarial parasites or even fungi rather than representing autoimmune response. The question of whether an antitoxic immune response to malarial GPI provides protection against malaria remains open. However, the novel microarray-based approach reported here addresses most technical challenges that have hampered previous work and allows for a more accurate analysis of samples.

Author contributions

F.K., G.P. and P.H.S. designed the research. F.K., M.T. and X.L. performed the research. X.L., Y.-U.K. and F.T. contributed new reagents and analytic tools. F.K., M.T., G.P. and P.H.S. analyzed the data. F.K., M.T., X.L., G.P. and P.H.S. wrote the paper with contributions from the other authors.

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Figures

Figure 1. Synthetic GPI glycans for microarray construction. Seven synthetic GPI glycans (1-7) were arrayed as quadruplicates in 64 identical screening units on a glass slide coated with bovine serum albumin to build a GPI glycan microarray.

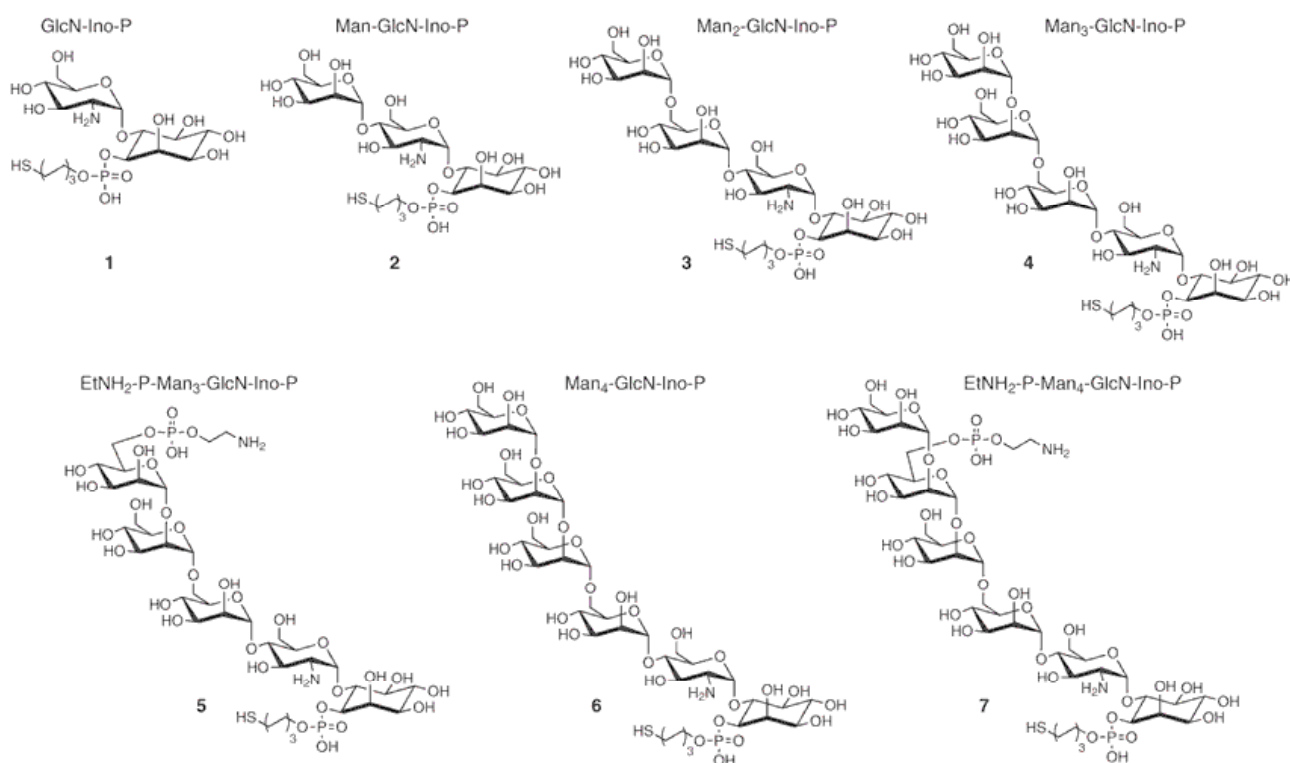
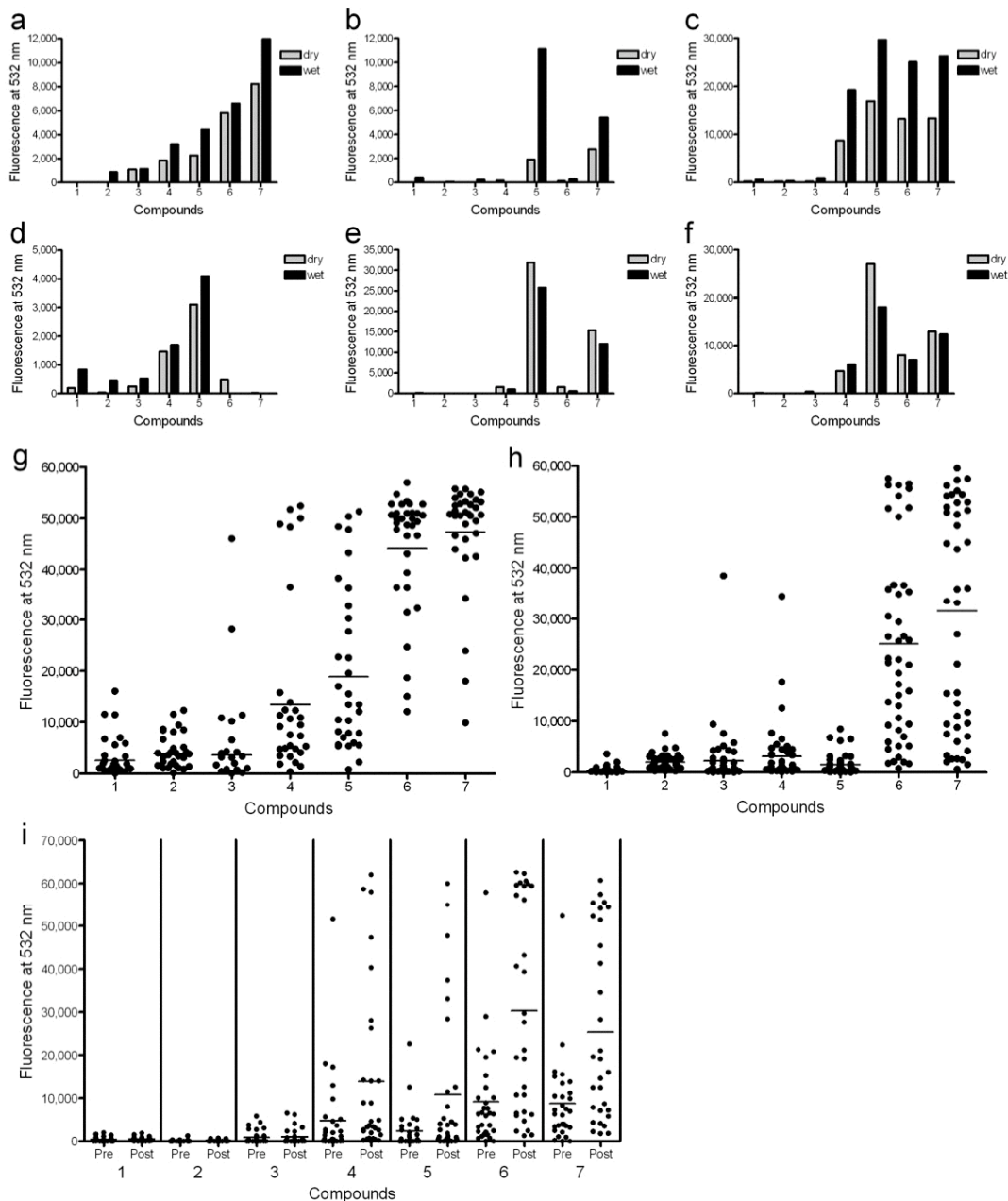
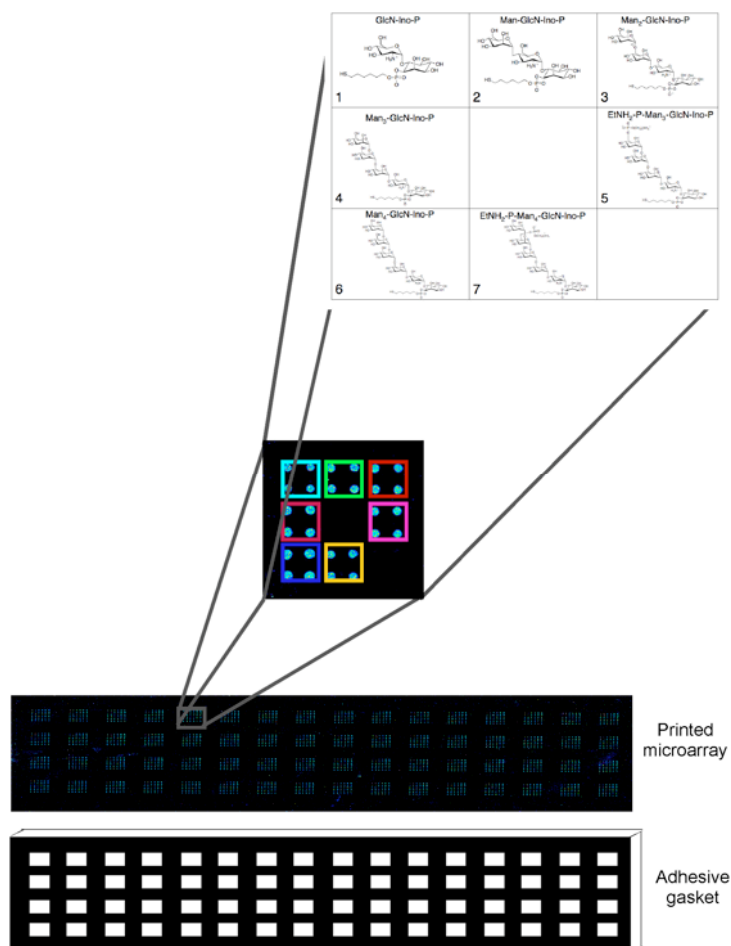


Figure 2. Analysis of serum samples using synthetic GPI glycan microarray. (a–f) Temporal stability of individual anti-GPI IgG response patterns in donors living in a malaria hyperendemic region of Ghana. Representative results from six donors are shown to demonstrate the diversity and temporal stability of the fine specificity of individual anti-GPI responses. Paired samples from blood donations of the same donor taken at the end of the low-transmission dry season (dry) or at the end of the high-transmission wet season (wet) are compared. (g,h) IgG levels against synthetic GPI compounds 1-7 in sera from cohorts of African donors living in a malaria-endemic area (g) and from malaria non-exposed Europeans (h). Each data point represents one serum; bars indicate the mean antibody level. (i) Increase in mean serum IgG levels against synthetic GPI compounds after experimental *P. falciparum* sporozoite challenge of malaria non-exposed Europeans. Sera were taken before (Pre) or after (Post) experimental challenge. Each data point represents one serum; bars indicate the mean level.

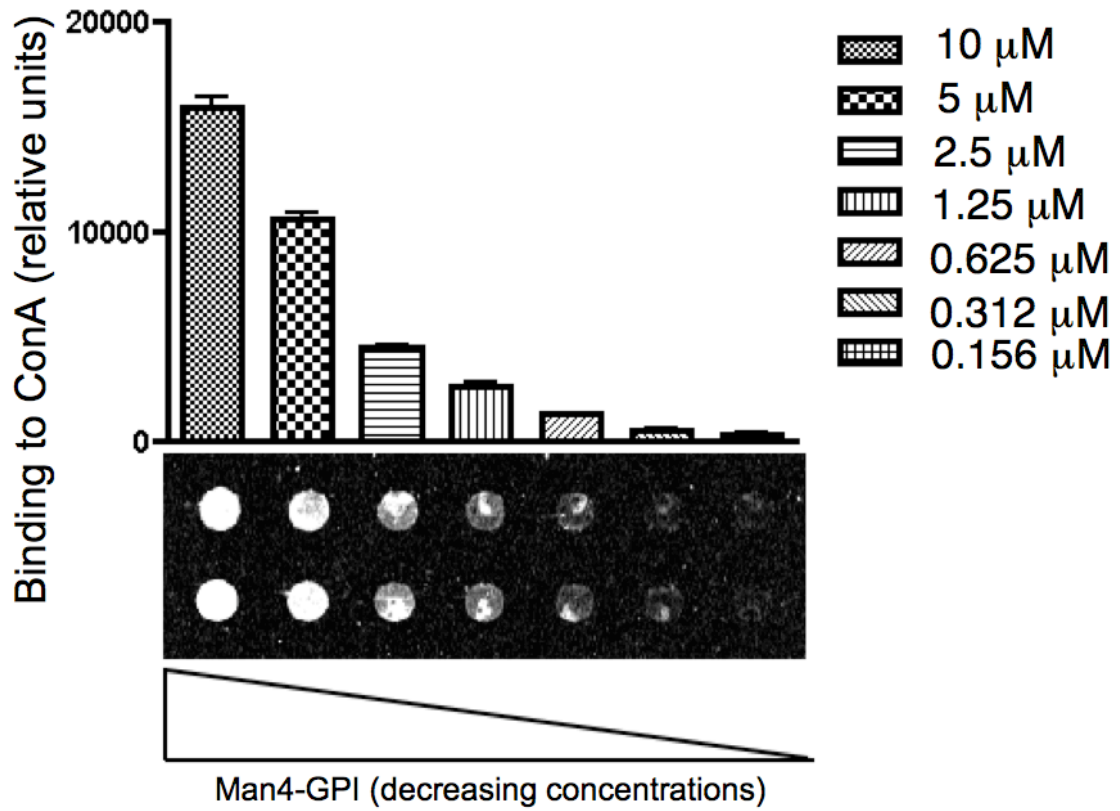


Supporting Information

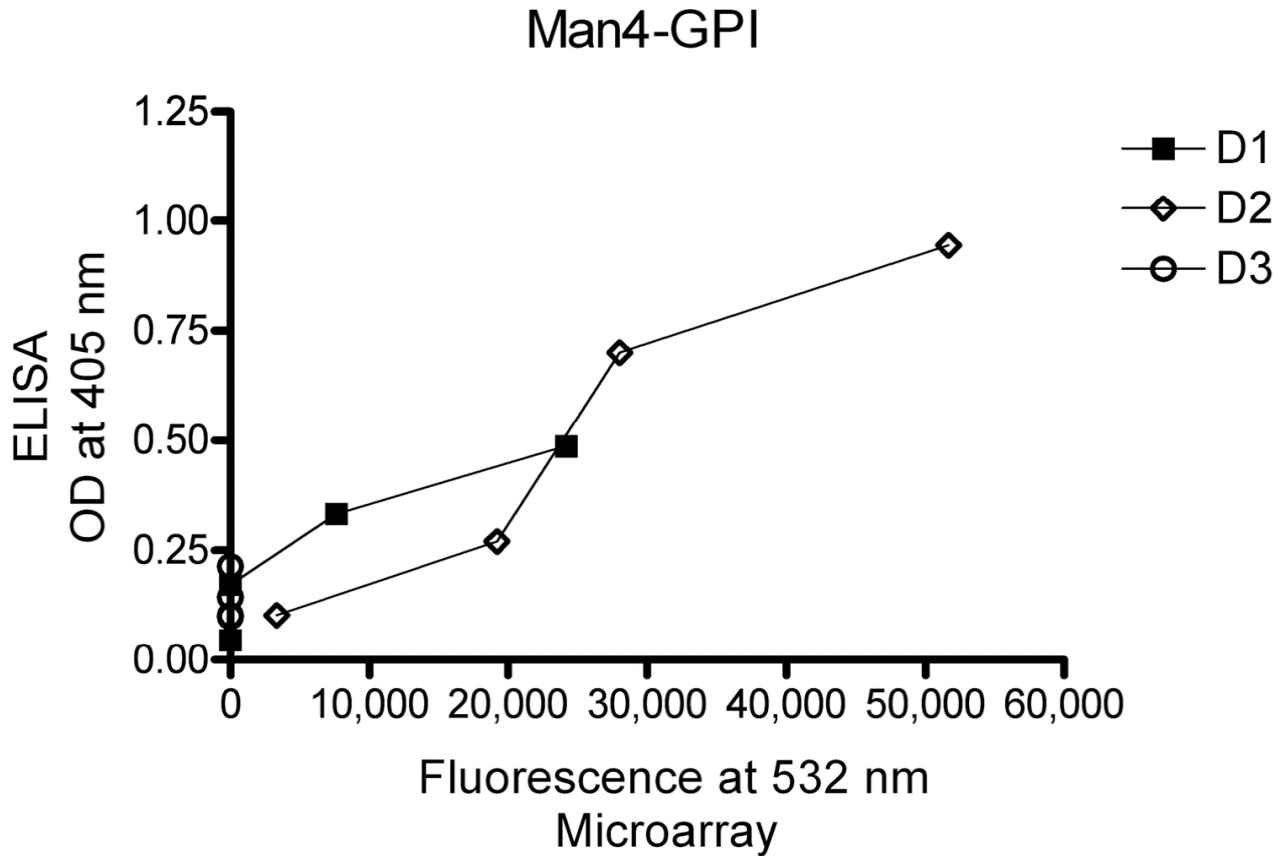
SI-Fig. 1: An adhesive gasket containing 64 chambers was adapted to the printed glass slide to build a 64-well screening device where the bottom of each well contains a single screening unit.



SI-Fig. 2: Validation of array printing. Binding of FITC-labeled ConA to a series of decreasing GPI 6 concentrations. Error bars represent standard deviations from paired data.



SI-Fig. 3: Validation of the microarray data by comparative serum titration experiments in ELISA versus microarray-based analysis. Shown are results obtained with serial dilutions of human sera (1:100; 1:300; 1:900; 1:2700) from three selected malaria-exposed donors (D1: low responder; D2: high responder; D3: non responder) that were analysed in parallel in GPI 7 immobilized ELISA plates and on a microarray slide.



Supplementary Subjects, Materials & Methods Section

Carbohydrate synthesis: Carbohydrates with a terminal sulfhydryl-containing linker were prepared as previously described¹. The terminal sulfhydryl-containing linker provided a handle for covalent immobilization of the structures to a maleimide-modified surface.

Functionalization of slides: Microarray slides were functionalized as previously described², briefly aldehyde slides (Genetix) were immersed in phosphate buffered saline (1xPBS, 50 mL) containing 1% BSA (w/v) and incubated overnight at room temperature. The slides were rinsed twice with distilled water (100 mL) and twice with 95% ethanol (50 mL), then dried under a stream of dry argon. Subsequently, the slides were immersed in anhydrous DMF (45 mL; Aldrich) containing 6-Maleimidohexanoic acid Nhydrosuccinimide ester (40 mg; Aldrich) and N, N-diisopropylethylamine (100 mM; Fluka). The slides were incubated in this solution for 24 h at room temperature, washed four times with 95% ethanol (50 mL), and stored in a vacuum dessicator until use.

Printing and validation of slides: A volume of 3 nL of a 10 mM solution of synthetic GPI-glycan in PBS were deposited on the surface of the slide to form each microarray spot using a Genetix array printer (Genetix). The slides were subsequently incubated in a humid chamber for 24h before being quenched with 1mM b-mercaptoethanol for 2h at room temperature. The slides were then washed three times with water and stored in a dessicator. Stored slides were stable for at least two months without changes in the reproducibility of the experiments.

To validate the printing of carbohydrates on the microarray, decreasing concentrations of **7** were immobilized and subsequently incubated with FITC-labelled ConA. A linear correlation between the concentration of GPI-Man₄ (**7**) and binding to ConA was observed to confirm the immobilization (see SI-Fig. 2). Comparative serum titration experiments in ELISA versus microarray-based analysis, performed with 12 selected sera, validated the microarray data (see SI-Fig. 3: for prototypical data).

Enzyme-linked immunosorbent assay (ELISA): Reacti-BindTM maleimide activated clear strip plates (PIERCE; Rockford, USA) were coated with 100 mL of a 50 mM solution of synthetic GPI. Wells were then blocked with 5% milk powder in PBS for 1 h at room temperature followed by three washings with PBS containing 0.05% Tween-20. Plates were then incubated with three-fold serial dilutions of human serum in PBS containing 0.05% Tween-20 and 0.5% milk powder for 2 h at room temperature. After washing, the plates were incubated with alkaline phosphatase-

conjugated F(ab')₂ fragment goat anti-human IgG (γ -chain specific) antibodies (Jackson ImmunoResearch Laboratories, West Grove, USA) for 1 h at room temperature and then washed. Phosphatase substrate (1 mg mL⁻¹ p-nitrophenyl phosphate (Sigma)) in buffer (0.14% Na₂CO₃, 0.3% NaHCO₃, 0.02% MgCl₂, pH 9.6) was added and incubated at room temperature. The optical density (OD) of the reaction product was recorded after appropriate time at 405 nm using a microplate reader (Titertek Multiscan MCC/340, Labsystems, Finland).

Subjects: Control (pre-immune) sera from malaria naive individuals were obtained from healthy adult Caucasian volunteers aged 18-45 years from the Basel area participating in a Phase I clinical trial approved by the Basel Research Ethics Committee (Genton et al, in press). The effect of sporozoite challenge was analyzed with sera from healthy malaria naive adult Caucasian subjects aged 18–50 years from the Oxford area recruited for a vaccine trial (Thompson, FM et al., submitted). Volunteers underwent experimental challenge with the chloroquine-sensitive 3D7 strain of *P. falciparum* through bites by infected laboratory-reared *Anopheles stephensi* mosquitoes. The study received ethical approval from the Oxfordshire Research Ethics Committee. Serum samples from malariaexposed individuals were taken from adult African volunteers aged 18-50 years and serial serum samples came from 9–22 years old volunteers from the Kassena Nankana District of northern Ghana.

Analysis of sera: Spotted microarray slides were covered with FlexWell-64 (GRACE BIO-LABS) layers. Wells were then blocked with 5% milk powder in PBS for 1 h at room temperature followed by three washings with PBS containing 0.05% Tween-20. The wells were subsequently incubated with human serum diluted 1:100 in PBS containing 0.05% Tween-20 and 0.5% milk powder for 2 h at room temperature. After washing, the slides were incubated with CyTM3-conjugated affinity-pure F(ab')₂ fragment goat anti-human IgG (Fc γ -fragment specific) antibodies (MILAN ANALYTICA AG) for 1 h at room temperature and then washed. Dried slides were read on a GenePixTM Personal 4100A (Axon Instruments) Microarray-scanner at a wavelength of 532nm. The resulting picture was quantitative analysed with GenePixTM Pro 6 software. Background fluorescence intensity from spotted buffer without carbohydrates was subtracted to GPI signals for each individual microarray slide.

Supplementary Information References

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**CHAPTER 4: SYNTHETIC GLYCOSYLPHOSPHATIDYLINOSITOL
MICROARRAY REVEAL DIFFERENTIAL ANTIBODY
LEVELS AND FINE-SPECIFICITIES IN CHILDREN
WITH MILD AND SEVERE MALARIA**

Manuscript in preparation

**SYNTHETIC GLYCOSYLPHOSPHATIDYLINOSITOL MICROARRAY
REVEAL DIFFERENTIAL ANTIBODY LEVELS AND FINE-
SPECIFICITIES IN CHILDREN WITH MILD AND SEVERE
MALARIA**

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Abstract

Glycosylphosphatidylinositol (GPI) glycolipids abound on the cell surface at the merozoite stage of *Plasmodium falciparum* life cycle and are a central toxin in malaria. The level at which the host adaptive immune response to GPI contributes to protection against malaria pathology remains unclear. Attempts to correlate anti-GPI antibody levels and pathology yielded contradictory results. Here, we present the application of a novel carbohydrate microarray based on synthetic *Pf*GPI glycans to assess levels and fine specificities of anti-GPI responses in healthy and malaria diseased individuals as well as the age dependent development of anti-GPI responses in malaria exposed children. Anti-GPI antibodies were only rarely found in children under the age of 18 months. Sera from subjects with severe malaria and healthy children contained antibodies that recognized predominantly synthetic Man₃-GPI and Man₄-GPIs. In contrast, an inverted length-dependent fine specificity of antibodies to GPI-glycans was observed in children with mild malaria. These sera also recognized substantially the most truncated GPIs included in the analysis, glycans comprising glucosamine-inositol moieties without or with one or two coupled mannose residues. This sub-population of anti-GPI antibodies may be involved in protection against the development of severe malaria pathology.

Introduction

Glycosylphosphatidylinositols are evolutionary conserved glycolipids found in the outer cell membranes of virtually all eukaryotic cells from vertebrates to protozoa (1) and constitute up to 90% of protein glycosylation in protozoan parasites (2). The glycan part of the GPI glycolipid consists of a conserved, linear 6-*O*-(ethanolamine-PO₄)- α -Man-(1-2)- α -Man-(1-6)- α -Man-(1-4)- α -GlcNH₂-(1-6)-*myo*-inositol-1-PO₄ core structure. Although evolutionarily conserved, species-dependent branching of the GPI core structure results in unique GPI molecules comprising epitopes that can be recognised by the mammalian immune system (3). GPIs anchor a diverse range of proteins to the surface of *Plasmodium falciparum*, but may also exist free of protein attachment (4-6). As for other parasitic diseases, such as trypanosomiasis and toxoplasmosis, certain pathogenic features may be caused by parasite GPI-glycans (7, 8). *In vitro* and *in vivo* studies have established plasmodial GPI as potent activator of the host immune system and as inducer of pro-inflammatory cytokines (9-11). Although inflammatory cytokines are beneficial in controlling and eliminating parasites, excessive production of these cytokines, particularly TNF- α , is thought to play a significant role in aggravating clinical symptoms which may lead to severe malaria.

In populations exposed to *P. falciparum*, the antibody response to purified GPIs is characterized by an age-dependent development that correlates with the acquisition of immunity to severe malaria (12). Therefore it has been postulated that antibodies against plasmodial GPI mediate anti-toxic and anti-disease immunity against malaria and that GPI could be a suitable component of a malaria vaccine. In the present report we show the application of a novel carbohydrate microarray based on synthetic *Pf*GPI glycans to assess levels and fine specificities of anti-GPI responses in healthy and malaria diseased individuals as well as the age dependent development of anti-GPI responses in Tanzanian children.

Material and Methods

Subjects. The study was conducted in Ifakara, Tanzania from June to September 2003 as described in (13). Samples were collected from children (aged 3 to 60 months) presenting with malaria at the hospital. Severe malaria cases were defined according to the World Health Organization criteria for severe malaria (14). Uncomplicated malaria was defined as the presence of asexual *P. falciparum*, an axillary temperature of $>37.5^{\circ}\text{C}$, or symptoms of headache or myalgia but no other signs of severe malaria. Exclusion criteria were confirmed coinfection, malnutrition (mid-upper-arm circumference [MUAC] of <12 cm), or antimalarial treatment during the last 14 days. Asymptomatic children (presence of *P. falciparum*, axillary temperature of $<37.5^{\circ}\text{C}$, and no other symptoms) were age-matched as closely as possible to patients by convenience sampling in the same area in 2006. Ethical clearance was obtained from the Ifakara Health Research and Development Centre's scientific review board and the Medical Research Coordinating Committee of the National Institute for Medical Research in Tanzania.

Synthetic GPI microarray. Carbohydrates with a terminal sulfhydryl-containing linker were prepared as previously described (15). The terminal sulfhydryl-containing linker provided a handle for covalent immobilization of the structures to a maleimide-modified surface. Functionalization and printing of slides were performed as previously described (3). Briefly aldehyde slides (Genetix Ltd, Hampshire, UK) were immersed in PBS containing 1% BSA (w/v) and incubated overnight at room temperature. The slides were rinsed twice with distilled water and twice with 95% ethanol, then dried under a stream of anhydrous argon. Subsequently, the slides were immersed in anhydrous DMF (Aldrich, St. Louis, Mo.) containing 1mg/mL 6-Maleimidohexanoic acid *N*-hydrosuccinimide ester (Aldrich, St. Louis, Mo.) and *N,N*-diisopropylethylamine (100 mM; Fluka, St. Louis, Mo.). The slides were incubated in this solution for 24 h at room temperature and washed four times with 95% ethanol. A volume of 3 nL of a 10 μM solution of synthetic GPI-glycan in PBS were deposited on the surface of the slide to form each microarray spot using a Genetix array printer (Genetix Ltd, Hampshire, UK). The slides were subsequently incubated in a humid chamber for 24h before being quenched with 1mM β -mercaptoethanol for 2h at room temperature. The slides were then washed three times with water and stored in a dessicator. Microarrays were validated with FITC-labelled ConA and comparative serum titration experiments in ELISA versus microarray-based analysis, yielding good correspondence of results (9). Stored slides were stable over two months without alteration in the reproducibility of the experiments.

Microarray-based immunoassay. Spotted microarray slides were covered with FlexWell-64 (GRACE BIO-LABS, Bend, OR) layers. Microarray wells were blocked with 0.5% milk powder in PBS for one hour at room temperature followed by three washings with PBS containing 0.01% Tween-20. Afterwards wells were incubated with human serum diluted 1:100 for two hours at room temperature. After washing, slides were incubated with indocarbocyanine dye-conjugated affinity-pure F(ab')₂ fragment goat anti-human IgG (Fc_γ-fragment specific) antibodies (MILAN ANALYTICA AG) for one hour at room temperature and washed again. Dried slides were read on a GenePix™ Personal 4100A (Axon Instruments, Sunnyvale, CA) Microarray-scanner at a wavelength of 532nm. The resulting picture was quantitatively analysed with GenePix™ Pro 6 software. Background fluorescence intensity from spotted buffer without carbohydrates was subtracted to GPI signals for each individual microarray slide.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Aliquots of lysed *P. falciparum* blood stage parasites were mixed with loading buffer (1.7 ml of 0.5 M Tris-HCl [pH 6.8], 2 ml of glycerol, 4.5 ml of 10% sodium dodecyl sulfate, 1 ml of β-mercaptoethanol, 0.8 ml of bromophenol blue [0.3%, wt/vol]) and heated 10 min at 95°C before loading on SDS-10% PAGE minigels. As a molecular weight marker, SeeBluePlus (Invitrogen) was used. Separated proteins were transferred electrophoretically to nitrocellulose filter (Protean Nitrocellulose, BA 85; Schleicher & Schuell) by semidry blotting. Blots were blocked with PBS containing 5% milk powder and 0.1% Tween 20 overnight at 4°C and then incubated with human serum diluted 1:200 for 2 h. After several washing steps, blots were incubated with goat anti-human IgG horseradish peroxidase conjugated Ig (Bio-Rad Laboratories, Hercules, Calif.) for 1 h. Blots were developed using the ECL system according to manufacturer's instructions.

Results

Effect of age on the acquisition of high anti-GPI IgG responses. Serum samples collected in Ifakara (Tanzania) from children aged 3-60 months (Table 1) with uncomplicated or severe malaria or healthy controls were analysed on synthetic GPI microarrays spotted with seven *Pf*GPI analogues (Fig. 1). Fluorescence values for the samples from small children aged 3-18 months (for GPI 6: median = 1708; range = 0-12531) were significantly lower than those for the samples from children aged 19-60 months (for GPI 6: median = 3700; range = 0-61301) (Mann-Whitney U, $P < 0.0001$) (Fig. 2). In addition, high anti-GPI IgG responses to all synthetic glycans were only rarely found in children under the age of 18 months, even in those with acute malaria infection. Regression analysis including all ages demonstrated a modest correlation between anti-GPI 6 and 7 antibodies and age in children with severe malaria ($r^2 = 0.125$, $P = 0.010$ for GPI 6; $r^2 = 0.096$, $P = 0.026$ for GPI 7) but not for the shorter glycans.

In accordance, while serum samples from healthy Tanzanian infants involved in a vaccine trial had no significant anti-GPI IgG antibody levels, sera from their mothers contained antibodies that bound to Man₃- and Man₄-GPIs (Fig. 3). In immunoblotting experiments with lysates of *P. falciparum* blood stage parasites, serum samples of healthy mothers stained series of protein bands over a broad range of molecular weights, indicating the presence of a strong anti-protein antibody response (Fig. 4A). In comparison to their mothers the infants showed low antibody levels to *P. falciparum* proteins.

Sera of a GPI seropositive mother and her GPI seronegative child taken at 2.5 months of age showed a comparable binding pattern in Western blot analysis (Fig. 4B). Five months later the humoral immune response of the child to *P. falciparum* lysates changed considerably to a novel response pattern. These results suggest that although the mother possessed naturally acquired anti-protein and anti-GPI antibodies, maternally transmitted *P. falciparum*-specific antibodies were predominantly directed against protein antigens.

Levels and fine specificities of anti-GPI responses in healthy and malaria diseased individuals. In microarray analysis sera from subjects with severe malaria and healthy children contained antibodies that recognized predominantly the GPIs 5, 6 and 7 (Fig. 5). Only for GPI 7 the mean level of IgG antibody was significantly higher in children with severe malaria than in healthy controls ($P = 0.0041$). In contrast, an inverted length-dependent fine specificity of antibodies to *P. falciparum*-related GPI-glycans was observed in children with mild malaria, since these sera also recognized substantially the most truncated GPIs (GPIs 1 to 4) without a phosphate ethanolamine group. While the mean level of IgG antibody to GPIs 1, 2, 3 and 4 was significantly (see Table 1 for

P values) higher in children with mild malaria than in children of the two other cohorts, the mean fluorescence values to GPIs 5, 6 and 7 were significantly (see Table 1 for *P* values) higher for samples from children with severe malaria than for samples from subjects with uncomplicated malaria.

Selected GPI positive and negative serum samples from the three categories of individuals were analysed in Western blot analysis with total lysates of unsynchronized *P. falciparum* blood stage parasites. Serum samples that had microarray fluorescence signals less than 2000 for all synthetic GPI glycans were considered as GPI seronegative. All tested GPI seropositive samples yielded person-specific multiple band staining patterns independent of their clinical condition (Fig. 6). Reactivity and patterns obtained with serum samples from healthy GPI seronegative children were comparable to those of GPI responder. In contrast, mild and severe malaria diseased GPI seronegative children had lower antibody levels to *P. falciparum* lysates, especially children with uncomplicated malaria showed a markedly reduced anti-protein response.

Discussion

GPI of *P. falciparum* may be involved in the pathogenesis of malaria. Attempts to correlate anti-GPI antibody levels and pathology using purified GPI from cultured parasites have yielded contradictory results (12, 16, 17). Assuming that the preparations used were pure and free from contaminants, this inconsistency in results may reside in the fact that GPIs are heterogeneous and difficult to isolate from their natural sources. Therefore, anti-GPI antibody levels measured with extracted GPI critically depend on the ratio of Man₃- and Man₄-GPI in the antigen preparations. Concerns over the purity of GPI preparations derived from *P. falciparum* can be effectively abolished using fully synthetic GPIs for a more accurate analysis of serum samples. We are applying carbohydrate microarrays based on synthetic *P. falciparum* GPI to study antibody responses to different structural elements of this complex antigen. In a previous study, microarray-based serological analyses with synthetic GPIs demonstrated differences in the fine specificity of anti-GPI antibodies in malaria-exposed and non-exposed adult populations, indicating that anti-Man₃ GPI responses are primarily elicited by malaria parasites, while anti-Man₄ GPI antibodies may arise in response to exposure to a variety of pathogens (3).

In this study we applied the novel and validated carbohydrate microarray to assess levels and fine specificities of anti-GPI responses in healthy and malaria diseased individuals. Sera from Tanzanian subjects with severe malaria and healthy children contained antibodies that recognized predominantly Man₄-GPIs and Man₃-GPI with a phosphate ethanolamine group. Strikingly, a significant different antibody response was found in patients with mild malaria, since these sera recognized substantially the most truncated GPIs without a phosphate ethanolamine group. Presence of these antibodies in a proportion of subjects with mild malaria and lack in children with severe disease may suggest that this sub-population of anti-GPI antibodies is involved in protection against the development of severe malaria pathology. Anti-Man₄ antibodies are present in malaria unexposed as well as exposed populations and it is possible that these antibodies recognize functionally irrelevant epitopes, predominantly protein-bound sub-population of parasite GPI molecules.

P. falciparum GPIs occur at relatively high levels both as moieties that anchor proteins and as glycolipids not attached to proteins (free GPI) (6). The fine specificity of monoclonal antibodies (mAbs) produced against synthetic GPI and their reactivity with parasite expressed GPI in immunofluorescence analysis (IFA) and with GPI anchors of parasite protein in Western blotting correlated (18). Intense staining of *P. falciparum* and *Trypanosoma brucei* parasites in IFA and Western blotting analysis was only obtained with mAbs that also bound strongly to the most truncated GPI-1, the glycan comprising glucosamine-inositol moieties without mannose residues.

GPI-specific mAbs recognising more complex epitopes with mannose sugars coupled to the glucosamine-inositol moiety showed reduced binding to parasite-derived GPI in IFA and Western blotting, probably due to sterical hindrance. Thus, naturally occurring antibodies cross-reactive with truncated GPIs found only in children with uncomplicated acute malaria may have better access to parasite GPIs, resulting in more effective anti-GPI neutralizing activity.

Acquired immunity to malaria relates also to IgG responses against protection-associated protein antigens. Therefore selected serum samples of the three categories of individuals in the same age range were analysed in Western blot analysis with lysates of *P. falciparum* blood stage parasites. Interestingly, GPI seropositive samples independent of their clinical condition correlated with a substantial anti-protein IgG response. In contrast to GPI seronegative healthy children, seronegative mild and severe malaria diseased individuals had lower antibody levels to *P. falciparum* lysates. These results indicate, that an acute malaria infection decreases at some stage serum antibody levels due to binding of parasite-specific immunoglobulins to their antigens.

Although regression analysis demonstrated only modest correlations between GPI specific IgG levels and age, high anti-GPI antibody responses were only rarely found in children under the age of 18 months, even in those with acute malaria infection. It is well known that the immune system of children under two years of age has a reduced capacity to produce antibodies to certain carbohydrate antigens. In humans IgG molecules of all subclasses, but with different efficiencies, cross the placenta and confer passive immunity to the newborn (19, 20). In malaria endemic areas, young children are thought to be protected against malaria attack during the first few weeks of life partially by transplacentally acquired antibodies (19). Our results indicate that GPI specific IgGs are less effective maternally transmitted than anti-protein antibodies, which may contribute to the relative protection by killing the parasite or by reducing replication and hence parasite burden.

The synthetic GPI microarray-based approach allowed more differentiated insights than could be obtained with extracted GPI for the role of anti-GPI antibodies in antitoxic immunity to malaria. Our results argue for a protection-associated role of a sub-population of anti-GPI IgGs cross-reactive with truncated *Pf*GPI glycans against the manifestations of severe malaria. Thus, glucosamine-inositol moieties coupled with one or two mannose sugars may be the functional relevant epitopes accessible for protective anti-GPI antibodies. However, a direct role for anti-GPI antibodies of the same or another fine specificity in neutralizing parasite toxins involved in malaria pathogenesis remains to be demonstrated. The observations summarized here are of major significance for the design of a GPI-based anti-toxic malaria vaccine candidate aiming to reduce childhood mortality in malaria endemic regions.

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Figures

Fig. 1. A: Structures of the seven synthetic GPI-glycans (1-7) that were printed on a BSA-coated glass slide to build a GPI-glycan microarray. B: Example microarray image after incubation with human serum diluted 1:100. C: Spotting order of the seven GPI-glycans that were arrayed as quadruplicates in 64 identical screening units on a single slide.

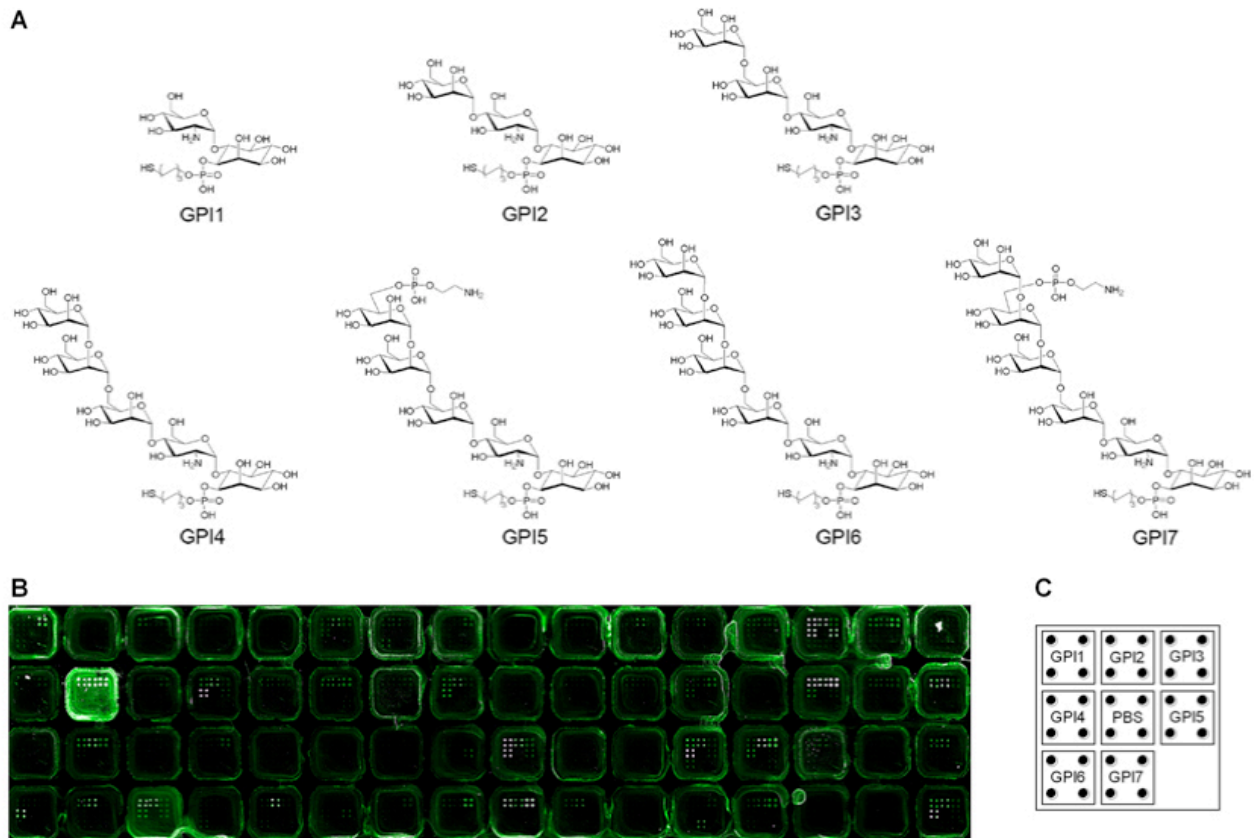


Fig. 2. Effect of age on the acquisition of high anti-GPI IgG responses. Shown are anti-GPI-6 IgG levels from Tanzanian children with severe or mild malaria or healthy controls. Data are expressed as absolute fluorescence at 532 nm for samples at a serum dilution of 1:100.

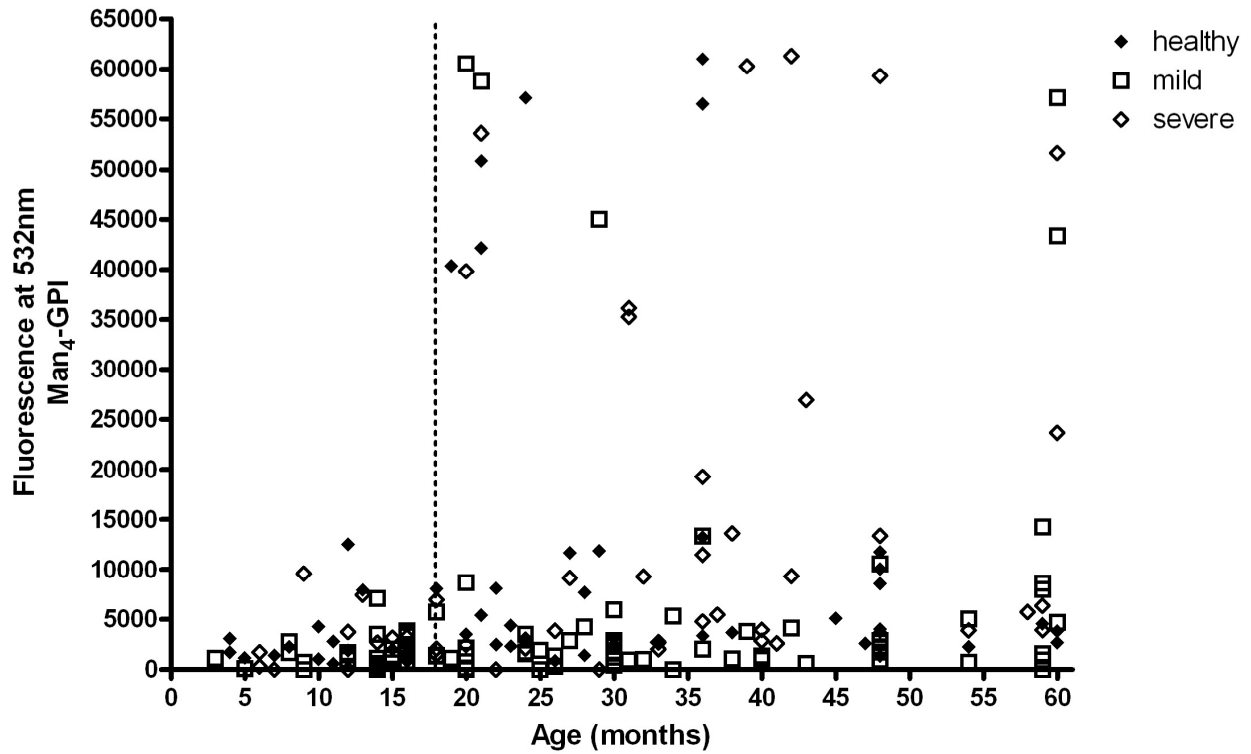


Fig.3. Anti-GPI-6 responses of healthy Tanzanian infants and mothers. Shown are IgG levels expressed as fluorescence at 532 nm in serial serum samples from infants aged 2.5, 7.5, and 9 months and mothers diluted 1:100. Horizontal lines indicate mean levels of anti-GPI response.

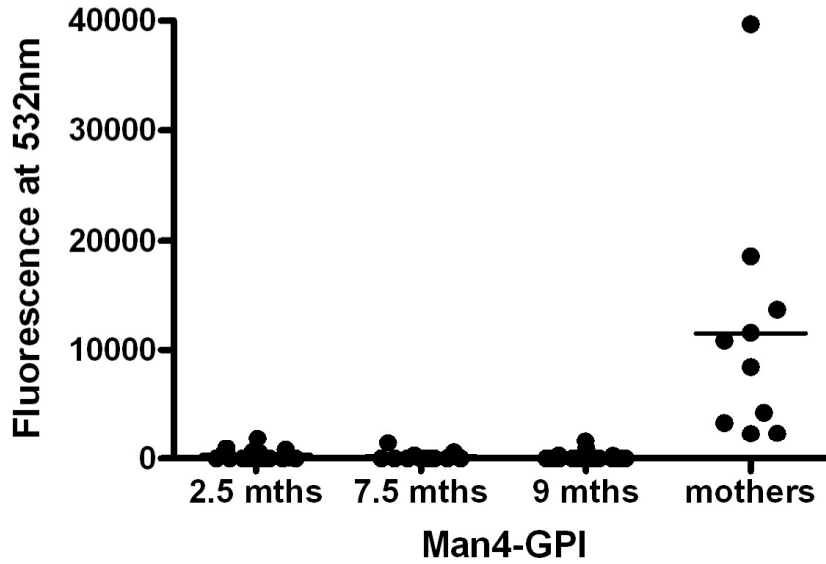


Fig. 4. A: Western blotting analysis with serum samples from healthy Tanzanian infants aged 2.5, 7.5, and 9 months and mothers using parasite lysates of *P. falciparum* blood stage cultures. Each lane represents serum from one individual donor at a dilution of 1:200. B: Immunoblotting analysis of sera of a mother and her child at 2.5 and 7.5 months of age.

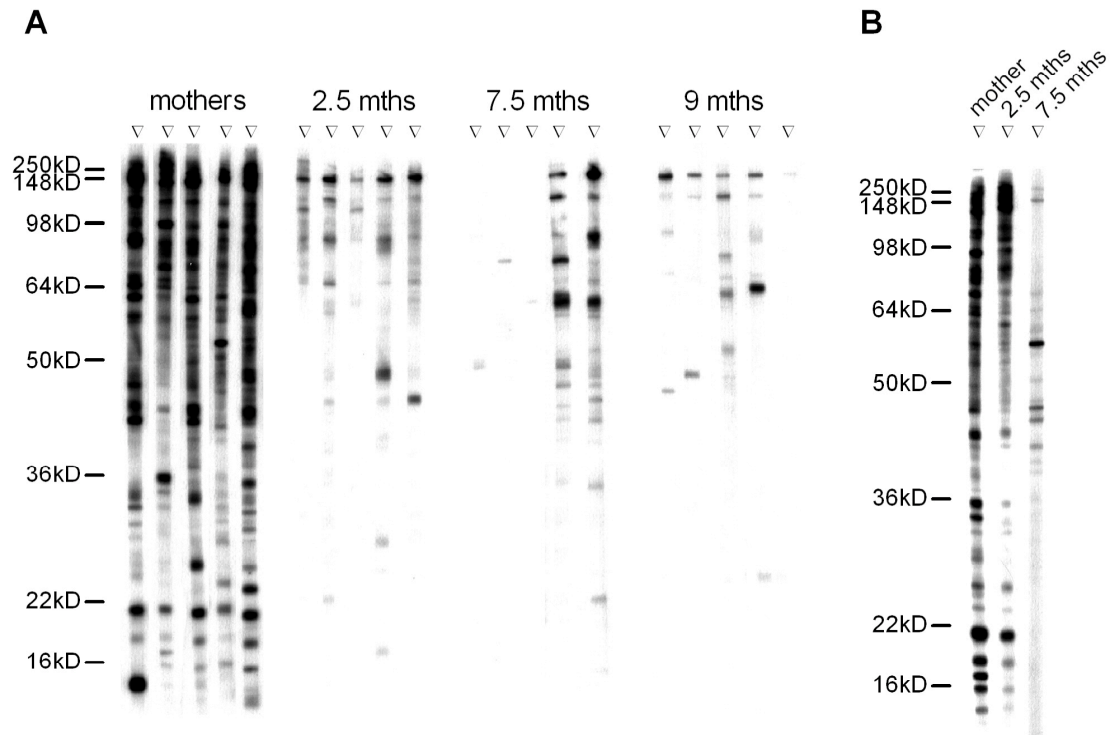


Fig. 5 Anti-GPI IgG levels and fine specificities of children with severe or uncomplicated malaria or healthy controls. Shown are IgG responses to each synthetic GPI compound expressed as fluorescence at 532 nm in serum samples diluted 1:100 from Tanzanian children with severe (s) or uncomplicated (m) malaria or healthy (h) controls. Horizontal lines indicate mean levels of anti-GPI response.

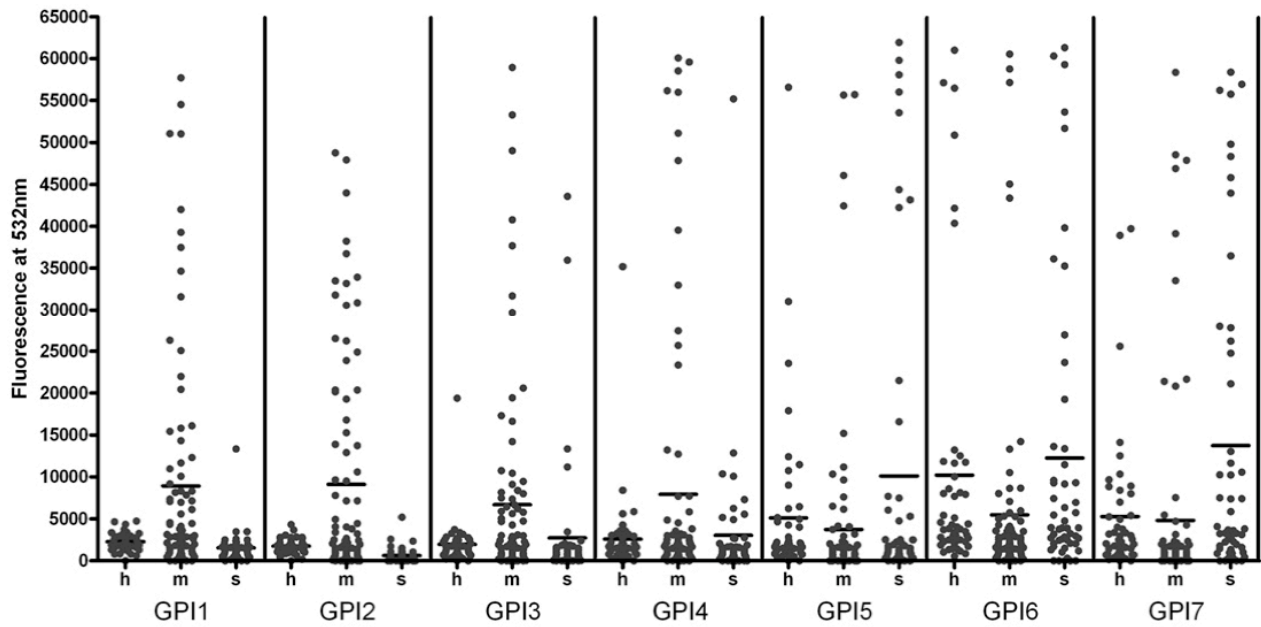
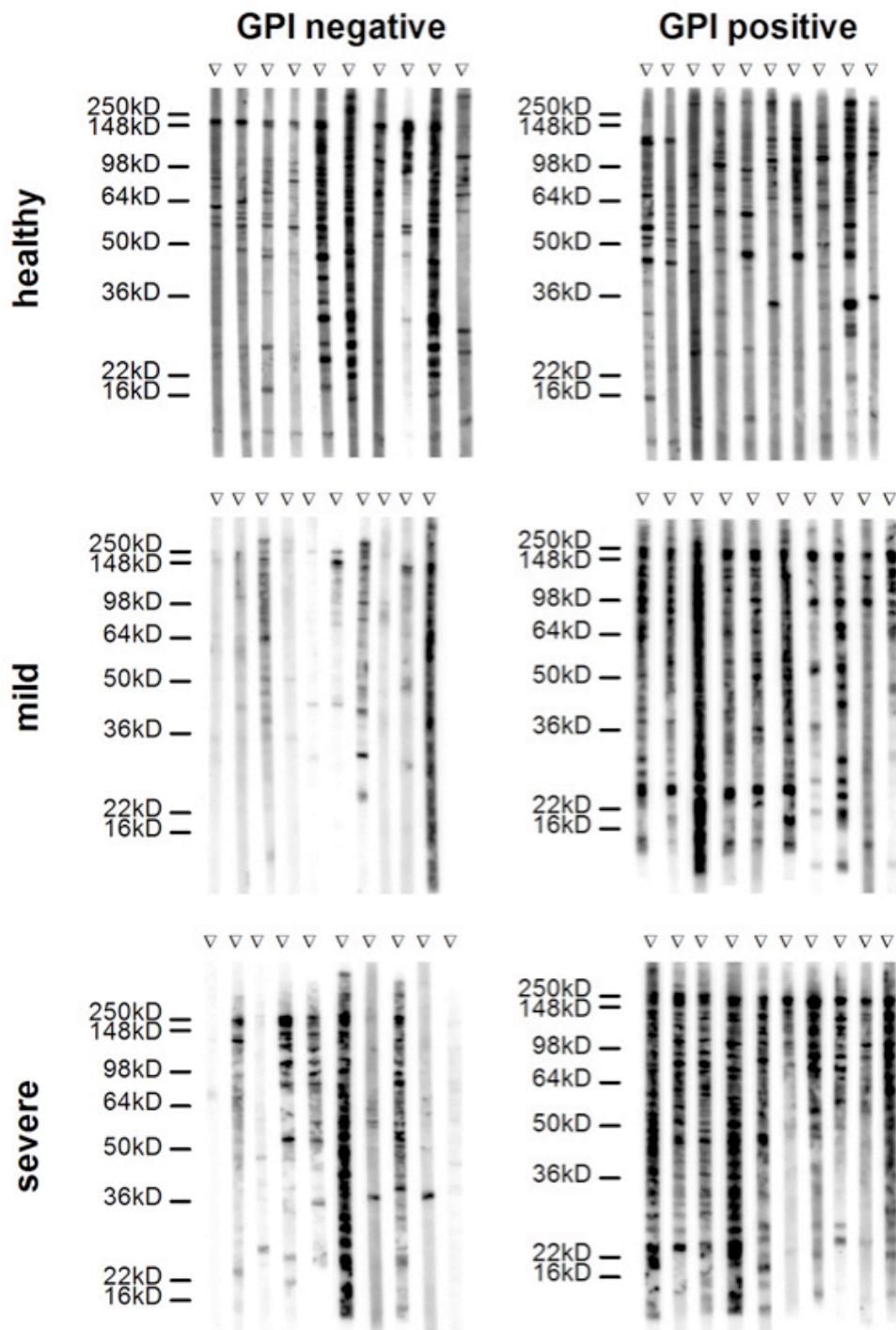


Fig. 6. Western blot analysis of the reactivity of sera from children with severe or mild malaria or healthy controls with *P. falciparum* K1 blood stage lysates. Each lane represents serum from one individual GPI seronegative or seropositive donor. All serum samples were used at a dilution of 1:200.



Chapter 4: Anti-*Pf*GPI antibodies in antitoxic immunity to malaria

Table 1. Anti-GPI IgG responses of Tanzanian children with severe or uncomplicated malaria or healthy controls

Condition	No. of children	Median age in mths (range)	anti-GPI IgG antibodies ¹				comparisons ²		
			GPI	mean	median	range	h-vs-m	h-vs-s	m-vs-s
healthy	50	24 (4-60)	GPI-1	2267	2177	0-4742	0.0008		
mild	87	26 (3-60)	GPI-1	8929	2855	0-57708		n.d.	
severe	52	29 (6-60)	GPI-1	1545	1333	0-13364			0.0002
healthy			GPI-2	1770	1743	0-4339	0.0001		
mild			GPI-2	9095	1952	0-48747		n.d.	
severe			GPI-2	628	427	0-5214			0.0001
healthy			GPI-3	1944	1345	0-19379	0.0067		
mild			GPI-3	6706	1920	0-58944		0.4989	
severe			GPI-3	2741	738	0-43579			0.0361
healthy			GPI-4	2591	1506	0-35195	0.0224		
mild			GPI-4	7924	1676	0-60086		0.7259	
severe			GPI-4	3052	1046	0-55193			0.0408
healthy			GPI-5	5124	1736	0-56585	0.4429		
mild			GPI-5	3723	458	0-55705		0.0968	
severe			GPI-5	10112	1348	0-61930			0.0116
healthy			GPI-6	10219	3739	0-60994	0.0531		
mild			GPI-6	5480	1690	0-60553		0.539	
severe			GPI-6	12265	3929	0-61301			0.0082
healthy			GPI-7	5286	2156	0-39710	0.8095		
mild			GPI-7	4820	764	0-58363		0.0041	
severe			GPI-7	13731	3648	0-58389			0.0008

¹ Antibody responses are expressed in fluorescence at 532nm

² Two-sided t-test ($P < 0.05$) between healthy (h) children or children with mild (m) or severe (s) malaria; grey shaded when means are significant different

**CHAPTER 5: FUNCTIONAL ACTIVITIES OF MONOCLONAL
ANTIBODIES RAISED AGAINST SYNTHETIC
PLASMODIUM FALCIPARUM
GLYCOSYLPHOSPHATIDYLINOSITOL GLYCANS**

Manuscript in preparation

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RAISED AGAINST SYNTHETIC *PLASMODIUM FALCIPARUM*
GLYCOSYLPHOSPHATIDYLINOSITOL GLYCANS**

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Running title: Functional activities of anti-GPI mAbs

Abstract

Glycosylphosphatidylinositol (GPI) glycolipids of plasmodial parasites are involved in malaria pathogenesis and there is evidence that anti-GPI antibodies can protect against severe malaria. We are using synthetic GPIs to characterise humoral immune responses against this dominant malaria toxin. Here we have generated GPI-specific B-cell hybridomas from mice immunized with a synthetic GPI glycan conjugated to a carrier protein. When hybridoma cell culture supernatants were screened with microarrays spotted with seven complete or truncated versions of synthetic *P. falciparum* GPI, monoclonal anti-GPI antibodies (mAbs) with different antigen binding patterns were identified. In immunofluorescence assays intense staining of *P. falciparum* and *Trypanosoma brucei* parasites was only obtained with antibodies that also bound strongly to the most truncated structure included in the analysis, a glycan comprising glucosamine-inositol moieties without mannose residues. Cross-reactivity of the anti-GPI mAbs with GPI anchors of proteins of plasmodial and trypanosomal parasites was demonstrated by Western blotting analysis. In *P. berghei* and *T. brucei rhodesiense* mouse infection models passive immunisation with anti-GPI mAbs did not reduce parasite *in vivo* multiplication and no delay of fatalities was observed. This is the first detailed description of the properties of anti-GPI mAbs. These new research tools will facilitate characterisation of the GPI-anchored proteome of protozoan parasites.

Introduction

Malaria is a debilitating and frequently fatal disease of the tropics caused by parasites of the genus *Plasmodium*. *P. falciparum* is responsible for the vast majority of deaths from malaria.^[1] The complex live cycle of *P. falciparum* alternating between a female mosquito and the human host starts when sporozoites are injected during the bite of an infected mosquito. The sporozoites invade hepatocytes and after schizogony thousands of merozoites capable of invading red blood cells are released into the blood stream. Ring, trophozoite, schizont and merozoite stages are distinguished during the asexual blood stage developmental cycle. This stage is responsible for the major symptoms and pathology of malaria, the most serious being anaemia and cerebral malaria. In recent years, malaria has spread at an alarming rate owing to the increasing resistance of the parasite to drugs, and the resistance of mosquitoes to insecticides. Therefore, new approaches to combat malaria, such as vaccine development and/or discovery of novel therapeutic agents, are urgently needed.^[2-4]

Glycosylphosphatidylinositols (GPIs) are ubiquitous molecules that anchor proteins in the membrane of virtually all eukaryotic cells from protozoa to vertebrates^[5-8] and constitute up to 90% of protein glycosylation in protozoan parasites. The glycan part of the GPI glycolipid consists of a conserved, linear 6-*O*-(ethanolamine-PO₄)-Man- α (1-2)-Man- α (1-6)-Man- α (1-4)-GlcNH₂- α (1-6)-*myo*-inositol-1-PO₄ core structure. Although evolutionarily conserved, species-dependent branching of the GPI core structure results in unique GPI molecules comprising epitopes that can be recognised by the mammalian immune system.^[9]

The most prevalent form of protein glycosylation in malaria parasites is the C-terminal addition of a GPI membrane anchor.^[10] The surfaces of the various extra-cellular forms of the malaria parasite, the merozoite, gamete, ookinete, and sporozoite, are coated by different proteins that are either known or presumed to be GPI-anchored. Some of these proteins are well characterised including MSP-1 and MSP-2 on merozoites,^[11,12] Pfs48/45 on gametes,^[13] Pfs25 and Pfs28 on ookinetes,^[14,15] and CSP on sporozoites.^[16,17] Genomic and proteomic studies suggest that only a minority of the GPI-anchored surface proteins of this parasite have been identified so far.^[18-20] GPI-anchored proteins are crucially involved in host-parasite interactions and several of them are regarded as key candidate antigens for a multi-valent subunit malaria vaccine.

There is growing evidence that the GPIs of protozoan parasites display diverse biological activity and contribute to parasite-associated pathogenesis. Among the bioactive molecules produced by the *P. falciparum* parasites, GPI has emerged as a central toxin that induces the expression of many host genes implicated in malaria pathogenesis. In particular the induction of pro-inflammatory cytokines by GPIs appears to be an important element in the development of

severe malaria.^[21-23] Natural semi-immunity developed by individuals living in areas of high malaria transmission is thought to be a combination of anti-toxic and anti-parasitic immune responses and lack of anti-toxic immunity may represent a key factor responsible for the vulnerability of infants to severe malaria. Anti-GPI antibodies have been proposed as mediators of malaria “anti-disease” immunity since the age-dependence of their development correlates with the acquisition of immunity to severe malaria.^[24] Toxin-neutralisation assays using anti-GPI antibodies have yielded controversial results.^[25-27] However, the proof of concept for GPI as an anti-toxic component for a malaria vaccine was demonstrated with a vaccination strategy using fully synthetic GPI analogues that delayed malaria in a lethal rodent model.^[28]

In the present report we show the application of carbohydrate microarrays based on synthetic *Pf*GPI for the selection of GPI specific B-cell hybridomas. The monoclonal anti-GPI glycan antibodies produced represent a new research tool to evaluate biological activities of anti-GPI antibodies and to characterise the GPI proteome of *P. falciparum* and other apicomplexan parasites.

Results

Generation of *Pf*GPI specific monoclonal antibodies. Mice were immunized three times with 10µg of ImmunEasy™ (QIAGEN) formulated synthetic GPI 6 (Fig. 1) conjugated to KLH as carrier protein. Blood samples were taken pre-immune, three weeks after the second and third immunization, and analysed on microarrays spotted with seven synthetic *Pf*GPI analogues (Fig. 1). High anti-GPI IgG titers could be detected in all immunized mice after the second immunization for the compounds GPI 2 to 7 whereas reactivity of the immune sera with GPI 1 was lower (Fig. 2A). A major titer increase was measured after the third vaccination only for GPI 1. Preimmune serum samples showed no significant reactivity with the spotted *Pf*GPIs analogues.

Six hybridoma cell lines secreting GPI-specific IgG were identified by screening of culture supernatants with the synthetic-GPI based microarray (Fig. 2B). The mAbs MTG2, MTG3, MTG5 and MTG6 reacted in microarray analysis with comparable fine specificity. These mAbs showed strong binding to all GPI-glycans, indicating that the glucosamine-inositol moieties include the most essential parts of their epitopes. Reactivity of the mAb MTG4 was low for the GPI 1 compound, indicating that the first mannose sugar coupled to the glucosamine-inositol moieties is needed in addition for a strong binding. The fine-specificity of the mAb MTG1 was unique, since stronger binding to *Pf*GPI 5 when compared to the other glycans was measured.

Anti-GPI mAbs bind to native GPI of *Plasmodium* and *Trypanosoma* parasites. All six anti-GPI mAbs were tested for parasite binding using an indirect immunofluorescence assay (IFA). The mAbs MTG2, MTG3, MTG5 and MTG6 showed strong binding to *P. falciparum* blood stage parasites in IFA, while mAbs MTG1 and MTG4 yielded only weak signals (Fig. 3). None of the anti-GPI mAbs showed binding to uninfected erythrocytes (not shown). In accordance, mAbs MTG2, MTG3, MTG5 and MTG6, but not mAbs MTG1 and MTG4, cross-reacted also strongly with *in vitro* cultivated *T. brucei rhodesiense* parasites (Fig. 3). As expected, an evenly distributed staining of the cells was observed, indicating a membrane-localised expression of the GPI-antigens accessible to the mAbs.

Expression of GPI-anchored proteins during blood stage development was analyzed by Western blotting with lysates of highly synchronised *in vitro* cultivated *P. falciparum* blood stage parasites harvested at different time-points. Individual samples thus represented ring, trophozoite or schizont stages. The anti-GPI mAbs MTG2, MTG3 and MTG6 but not mAbs MTG1, MTG4 and MTG5 (not shown) stained series of bands over a broad range of molecular weights (Fig. 4A, lanes 1-8). While a few bands dominated in the ring stage, new bands emerged in the trophozoite stage and the relative signal abundances of bands increased considerably, demonstrating specificity of the

anti-GPI mAbs for developmentally regulated GPI-anchored proteins. Uninfected erythrocytes yielded no detectable signal (not shown). Less complex band pattern were obtained with sporozoite lysates (Fig. 4A, lane 9). The same *P. falciparum* Western positive anti-synthetic-GPI mAbs stained multiple bands with cell lysates of *P. berghei* and *P. chabaudi* asexual blood stages harvested from infected mice (Fig 4B). With lysates of bloodstream-form trypanosomes cultivated in host-cell free medium a few distinct bands were stained with the anti-GPI mAbs (Fig. 4C).

Evaluation of parasite inhibiting activity of GPI specific mAbs. *In vitro* growth inhibition assays in absence of phagocytes and an active complement system were performed to assess parasite inhibitory activity of the anti-GPI mAbs with blood stage *P. falciparum* and with *T. brucei rhodesiense* parasites. Even at final antibody concentrations as high as 500 µg/mL the anti-synthetic-GPI mAbs showed no significant growth inhibitory effects in five independent experiments conducted with two different batches of antibody preparations (data not shown).

In vivo activity of the anti-GPI mAbs was assessed in outbred NMRI mice infected with either a GFP expressing *P. berghei* ANKA strain or with the human *T. brucei rhodesiense* strain STIB 900. mAbs (200µg) formulated in PBS were administered intravenously 24 h before and 72 h after infection. Parasitemia levels were not significantly different between test and control groups (Fig. 5A and 5C), and no delay of fatalities was observed in three independent experiments conducted with different batches of anti-GPI antibody preparations (Fig. 5B and 5D).

Discussion

Glycosylphosphatidylinositols are evolutionary conserved glycolipids found in the outer cell membranes of all eukaryotes. GPIs anchor a diverse range of proteins to the surface of *P. falciparum*, but may also exist free of protein attachment.^[29-31] As for other parasitic diseases, such as trypanosomiasis and toxoplasmosis, certain pathogenic features of malaria may be caused by parasite GPI-glycans.^[32,33] *In vitro* and *in vivo* studies have established plasmodial GPI as a potent activator of the host immune system and as inducer of pro-inflammatory cytokines.^[21-23] These responses seem to play a critical role in the pathogenesis of severe malaria. In populations exposed to *P. falciparum*, the antibody response to purified GPIs is characterized by an age-dependent development that correlates with the acquisition of immunity to severe malaria.^[24] Therefore, it has been postulated that antibodies against plasmodial GPI mediate anti-toxic and anti-disease immunity against malaria and that GPI could be a suitable component of a malaria vaccine.

The lack of a reliable method for the characterization of anti-GPI antibodies in large numbers of serum samples has made it difficult to generate strong supportive data for this hypothesis. Moreover many studies on GPI have been criticized, since incompletely characterised antigen preparations extracted from cultured parasites were used. Polysaccharides are often heterogeneous and difficult to isolate from their natural sources. Concerns over the purity of GPI preparations derived from *P. falciparum* can be effectively abolished if results can be repeated using fully synthetic GPIs. The use of pure fully characterized synthetic material allows for a direct correlation of the antibody signal to a specific glycan structure. We are applying carbohydrate microarrays based on synthetic *P. falciparum* GPI to study antibody responses to different structural elements of this complex antigen. Microarray-based serological analyses with synthetic GPIs demonstrated differences in the fine specificity of anti-GPI antibodies in malaria-exposed and non-exposed populations, indicating that anti-Man₃ GPI responses are primarily elicited by malaria parasites, while anti-Man₄ GPI antibodies may arise in response to exposure to a variety of pathogens.^[9]

Monoclonal antibodies to *Pf*GPIs are important tools to study the role of GPIs in the pathophysiology of malaria. Although the generation of monoclonal antibodies to purified GPIs was reported more than a decade ago,^[34] the details of this work have not been published. In the present report we show the application of the novel carbohydrate microarray based on synthetic *Pf*GPI for the selection of GPI specific B-cell hybridomas from mice immunized with pure synthetic GPI glycan.

The adjuvanted GPI 6 KLH conjugate was immunogenic in mice and elicited IgGs cross-reactive with all spotted synthetic GPI analogues. The fine specificity of the vaccine-induced

humoral immune response was different from naturally elicited anti-GPI IgGs, since serum antibodies reactive with the shortest GPI oligosaccharides **1 - 3** were only sporadically detected.^[9]

To investigate the role of GPI in malaria pathogenesis and for a detailed analysis of the humoral immune response, six GPI-specific mouse B-cell hybridomas with variable fine specificities were generated by screening hybridoma cell supernatants for GPI-crossreactivity in synthetic-GPI microarray analysis. The fine specificity of the anti-GPI mAbs and their reactivity to native antigen correlated. mAbs MTG2, MTG3, MTG5 and MTG6, in contrast to the mAbs MTG1 and MTG4, showed strong binding to *P. falciparum* blood stage parasites in IFA. While the IFA positive mAbs showed strong binding to the glucosamine-inositol moieties of the highly truncated GPI **1**, mAbs MTG1 and MTG4 recognising more complex epitopes with mannose sugars coupled to the glucosamine-inositol moiety yielded only weak IFA signals. Sterical hindrance may result in reduced binding to native GPI. It is not clear whether free GPI and protein bound GPI are both accessible for the anti-GPI mAbs on the parasite cell surface. Notably, the same *Plasmodium* IFA positive mAbs cross-reacted also with *in vitro* cultivated *T. brucei* bloodstream forms, which express a dense surface coat of GPI-anchored variant surface glycoprotein (VSG). Binding to native GPI occurred even though *T. brucei* VSG-GPIs have a strain dependent addition of branching oligogalactose side chain to the conserved core glycan.^[4]

The produced monoclonal anti-GPI antibodies represent a new research tool to characterise the GPI proteome of *P. falciparum* and other apicomplexan parasites and may help to identify new GPI-anchored proteins. Reactivity of the anti-synthetic-GPI mAbs for parasite-encoded GPI anchored antigens was demonstrated in Western blotting analysis for *P. falciparum* blood stages and sporozoites, *P. berghei* and *P. chabaudi* blood stages and *T. brucei* bloodstream forms. In these immunoblots the mAbs detected multiple protein bands supporting genomic and proteomic studies, which predicted that only a minority of GPI-anchored surface proteins of *P. falciparum* has been discovered so far.^[18-20] Focusing on the blood stages, proteomic analysis of proteins labelled with radioactive glucosamine identified GPI anchoring on 11 known proteins.^[35] Another nine lower abundance [³H]-glucosamine-labelled GPI-anchored proteins were also apparent at this life stage, but these remained unidentified. To predict the identity of these proteins as well as those present at other life stages a hidden Markov model, trained on *P. falciparum* sequences has been developed. This bioinformatics approach predicted that the *P. falciparum* genome encodes 30 GPI-anchored proteins.^[35] Since sporozoites cannot be cultivated *in vitro*, this approach cannot be applied for this development stage.

The ability of the generated anti-GPI mAbs to bind to parasite GPI raises to possibility that these reagents may eventually be used immunotherapeutically in malaria to inhibit parasite growth or to neutralize the toxic activities of GPI *in vivo*. To evaluate biological activities of our anti-GPI

mAbs, we performed functional analysis, including *in vitro* parasite growth inhibition assays and passive protection experiments in animal infection models. Despite binding to the protozoan parasite species, the anti-GPI mAbs had no effect on parasite replication *in vitro*. Currently, the correlation of *in vitro* growth inhibitory activities of antibodies with their potential protective capacity *in vivo* is incompletely understood. Therefore, we performed passive immunization studies in mice infected with either *P. berghei* or with *T. brucei rhodesiense*. The administered anti-GPI glycan mAbs had no effect on parasitemia levels and no delay of fatalities was observed.

Conclusions

These results do not rule out that anti-GPI antibodies of the same or another fine specificity have the potential to modulate pathomechanisms in severe forms of the disease, such as cerebral malaria. In a *P. berghei* rodent model of severe malaria active immunization with synthetic GPI glycan resulted in substantial protection against malarial acidosis, pulmonary oedema, cerebral syndrome and fatality, without reducing parasite levels.^[28] Synthetic GPI may thus represent a suitable component for a malaria vaccine aiming to reduce childhood mortality in malaria endemic regions.

Experimental Section

Mouse immunogenicity studies. Three mice carrying human immunoglobulin C γ 1 heavy and C κ light chain gene segments^[36] were immunized subcutaneously and intraperitoneal with synthetic GPI 6 antigen (Fig. 1) conjugated to keyhole-limpet-hemocyanine (KLH) formulated in ImmunEasyTM adjuvant (QIAGEN, Hombrechtikon, Switzerland). Starting on day 0, they received at three-weekly intervals three doses of 10 μ g conjugate.

Generation of anti-GPI monoclonal antibodies (mAbs). Three days before cell fusion an immunized animal received an intravenous booster injection with 10 μ g of GPI-KLH conjugate in phosphate-buffered saline (PBS). From the killed mouse the spleen was aseptically removed and a spleen cell suspension in IMDM was mixed with PAI mouse myeloma cells as a fusion partner. Spleen and myeloma cells in a ratio of 1:1 were centrifuged; after the supernatant was discarded, the pellet was mixed with 1 mL pre-warmed polyethylene glycol 1500 sterile solution. After 60s 10mL of culture medium were added. After 10 min cells were suspended in IMDM containing hypoxanthine, aminopterin, thymidine, and 20% fetal bovine serum (HAT) and cultured in 96-well tissue culture plates. Cells were cultured at 37°C with 5% CO₂ and fed five days later with 100 mL of HAT- medium. Cells secreting GPI-specific IgG were identified by screening culture supernatants with a synthetic GPI microarray-based immunoassay. Six hybridoma cell lines producing GPI specific mAbs were identified and cloned twice by limiting dilution. The mAbs, named MTG1, MTG2, MTG3, MTG4, MTG5 and MTG6, were purified from spent culture supernatant of the hybridoma clones by protein A affinity chromatography (HiTrap rProtein A FF, Amersham Biosciences, Piscataway, USA). Purified MAbs were dialyzed against PBS with Slide-A-Lyzer Dialysis Cassette (PIERCE, Rockford, USA), aliquoted, and stored at -80°C.

Synthetic GPI microarray. Carbohydrates with a terminal sulfhydryl-containing linker were prepared as previously described.^[37] The terminal sulfhydryl-containing linker provided a handle for covalent immobilization of the structures to a maleimide-modified surface. Functionalization and printing of slides were performed as previously described.^[9] Briefly aldehyde slides (Genetix Ltd, Hampshire, UK) were immersed in PBS containing 1% BSA (w/v) and incubated overnight at room temperature. The slides were rinsed twice with distilled water and twice with 95% ethanol, then dried under a stream of anhydrous argon. Subsequently, the slides were immersed in anhydrous DMF (Aldrich, St. Louis, Mo.) containing 1mg/mL 6-Maleimidohexanoic acid *N*-hydrosuccinimide ester (Aldrich, St. Louis, Mo.) and *N,N*-diisopropylethylamine (100 mM; Fluka, St. Louis, Mo.). The slides were incubated in this solution

for 24 h at room temperature and washed four times with 95% ethanol. A volume of 3 nL of a 10 μ M solution of synthetic GPI-glycan in PBS were deposited on the surface of the slide to form each microarray spot using a Genetix array printer (Genetix Ltd, Hampshire, UK). The slides were subsequently incubated in a humid chamber for 24h before being quenched with 1mM β -mercaptoethanol for 2h at room temperature. The slides were then washed three times with water and stored in a dessicator. Microarrays were validated with FITC-labelled ConA and comparative serum titration experiments in ELISA versus microarray-based analysis, yielding good correspondence of results.^[9] Stored slides were stable over two months without alteration in the reproducibility of the experiments.

Microarray-based immunoassay. Spotted microarray slides were covered with FlexWell-64 (GRACE BIO-LABS, Bend, OR) layers. Microarray wells were blocked with 0.5% milk powder in PBS for one hour at room temperature followed by three washings with PBS containing 0.01% Tween-20. Afterwards wells were incubated with appropriate dilutions of mouse sera or hybridoma supernatants for two hours at room temperature. After washing, slides were incubated with indocarbocyanine dye-conjugated affinity-pure F(ab')₂ fragment goat anti-mouse IgG heavy-chain antibodies (Jackson ImmunoResearch Laboratories, West Grove, Pa.) for one hour at room temperature and washed again. Dried slides were read on a GenePix™ Personal 4100A (Axon Instruments, Sunnyvale, CA) Microarray-scanner at a wavelength of 532nm. The resulting picture was quantitatively analysed with GenePix™ Pro 6 software. Background fluorescence intensity from spotted buffer without carbohydrates was subtracted to GPI signals for each individual microarray slide.

mAbs Isotyping. The isotypes of the generated mAbs were determined by sandwich enzyme-linked immunosorbent assay (ELISA). ELISA microtiter plates Immunolon 4 (Dynex Technologies Inc., Chantilly, Va.) were coated with unconjugated sheep anti-human kappa (Serotec, Raleigh, NC) or goat anti-mouse lambda (Southern Biotech, Birmingham, Ala.) antibodies at 4°C overnight. Wells were blocked with 5% milk powder in PBS followed by three washings with PBS containing 0.05% Tween-20; then 100 μ L of tested supernatants were added to different wells, which were incubated and washed again. Plates were incubated for one hour with alkaline phosphatase-conjugated goat anti-mouse antibodies specific for IgG (Sigma, St. Louis, Mo.); IgG1; IgG2b; IgG3; IgM (Southern Biotech, Birmingham, Ala.) and with alkaline phosphatase-conjugated mouse anti-human antibodies specific for IgG1 (Southern Biotech, Birmingham, Ala.). After washing, phosphatase substrate was added and incubated at room temperature. The optical density of the reaction product was recorded at 405 nm.

Indirect immunofluorescence assay (IFA). *In vitro* cultivated *P. falciparum* or *T. brucei rhodesiense* parasites were washed and mixed with two volumes of a solution containing 4% paraformaldehyde and 0.1% Triton X-100. Droplets of 40 μ L of cell suspension were added to each well of a diagnostic microscope slide (Flow Laboratories, Baar, Switzerland) and incubated for 30 min at room temperature. Cells were blocked with blocking solution containing 100 mg/mL fatty acid-free bovine serum albumin in PBS. Immunostaining was performed by incubating the wells with 25 μ L of an appropriate hybridoma supernatant or purified mAbs dilution in blocking solution in a humid chamber for one hour at room temperature. After five washes with blocking solution, 25 μ L of 5- μ g/mL indocarbocyanine dye-conjugated affinity-pure F(ab')₂ fragment goat anti-mouse IgG heavy-chain antibodies (Jackson ImmunoResearch Laboratories, West Grove, Pa.), diluted in blocking solution containing 0.01 mg of Hoechst dye no. 33256 (Sigma, St. Louis, Mo.) per mL, were added to the wells and incubated for one hour at room temperature. Finally, the wells were washed five times, mounted with mounting solution (90% [vol/vol] glycerol containing 0.1 M Tris-Cl [pH 8.0] and 2 mg of o-phenylenediamine per mL) and covered with a coverslip. Antibody binding and DNA staining were assessed by fluorescence microscopy on a Leitz Dialux 20 fluorescence microscope and documented with a Leica DC200 digital camera system.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. For time course analyses, aliquots of synchronous cultures of *P. falciparum*-infected erythrocytes were harvested at 6-h intervals and washed with PBS. Pelleted infected red blood cells were lysed by mixing with a large volume (adjusted to 5% hematocrit) of 0.015% (wt/vol) saponin in 150 mM NaCl and 15 mM sodium citrate and incubated on ice. Finally, the pelleted parasites were resuspended in PBS buffer and stored at -80°C until further use. Aliquots of lysed *P. falciparum* blood stage parasites, *Anopheles stephansi* salivary gland lysate containing *P. falciparum* sporozoites, *P. berghei* (ANKA) and *P. chabaudi* blood stages harvested from infected mice, and *T. brucei* parasites were mixed with loading buffer (1.7 mL of 0.5 M Tris-HCl [pH 6.8], 2 mL of glycerol, 4.5 mL of 10% sodium dodecyl sulfate, 1 mL of β -mercaptoethanol, 0.8 mL of bromophenol blue [0.3%, wt/vol]) and heated 10 min at 95°C before loading on SDS-10% PAGE minigels. As a molecular weight marker, SeeBluePlus (Invitrogen, Basel, Switzerland) was used. Separated proteins were transferred electrophoretically to nitrocellulose filter (Protean Nitrocellulose, BA 85; Schleicher & Schuell) by semidry blotting. Blots were blocked with PBS containing 5% milk powder and 0.1% Tween 20 overnight at 4°C and then incubated with hybridoma supernatant or purified mAbs for 2 h. After several washing steps, blots were incubated with goat anti-mouse IgG horseradish peroxidase conjugated Ig (Bio-Rad Laboratories, Hercules,

Calif.) for 1 h. Blots were developed using the ECL system (PIERCE, Rockford, USA) according to manufacturer's instructions.

Parasite culture and in vitro growth inhibition assay. *P. falciparum* strain K1 was cultured essentially as described previously.^[38] The culture medium was supplemented with 0.5% AlbuMAX (Gibco, Paisley, Scotland) as a substitute for human serum.^[39] Synchronization of cultures was achieved by sorbitol treatment as described previously.^[40] Serogroup 0+ or A+ erythrocytes for passages were obtained from the Swiss Red Cross (Basel, Switzerland).

For *in vitro* growth inhibition assays, synchronous late trophozoites were diluted with fresh red blood cells to result in 0.5% parasitemia and mixed with purified mAb. The final hematocrit in cultures was adjusted to 0.5%. Each culture was set up in sextuplicate in 96-well flat-bottomed culture plates. After 96 h, the plates were centrifuged at $180 \times g$ for 5 min, and the culture supernatants were discarded. Pelleted erythrocytes were resuspended in 200 μ L of PBS supplemented with 15 μ g of hydroethidine fluorescent vital stain (Polysciences Inc., Warrington, Pa.) per mL and incubated at 37°C for 30 min. The erythrocytes were washed with PBS and analyzed in a FACSScan flow cytometer (Becton Dickinson, San Jose, Calif.) with CellQuest 3.2.1fl software. The hydroethidine emission was detected in the FL2 channel by logarithmic amplification, and the erythrocytes were gated on the basis of their forward and side scatters. A total of 30,000 cells per sample were analyzed. Percent inhibition was calculated from the geometric mean parasitemias of sextuplicate test and control wells as $100 \times [(control - test)/control]$. Statistical significance was calculated by a two-sided t test. Confidence intervals ($P < 95\%$) were calculated by antilogging the confidence limits calculated on the log scale.

Bloodstream form trypanosomes were cultivated in Minimum Essential Medium (MEM) with Earle's salts (GIBCO/BRL No 072-01100 P), supplemented with 25 mM HEPES, 1 g/L additional glucose, 2.2 g/L NaHCO₃, 10 ml/L MEM nonessential amino acids (100 \times) and heat inactivated horse serum (15%). The medium was further supplemented according to,^[41] with 0.2 mM 2-mercaptoethanol, 2 mM Na-pyruvate, 0.1 mM hypoxanthine and 0.016 mM thymidine. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air.

For *in vitro* growth inhibition assays, bloodstream form trypanosomes were inoculated into 96-well flat-bottomed microtiter plates (Costar, USA) at a density of 200 trypanosomes/100 μ L culture medium. The trypanosomes were incubated in the presence of purified mAbs for 72 h. Wells without antibodies served as controls and background fluorescence and absorbance of the mAbs containing medium were determined for each mAb. After incubation for 68 h, 10 μ L of the Alamar blue® dye was added into each well of the plate, which was then incubated under culture conditions for an additional 4 h. Fluorescence measurements were performed using a fluorescence plate reader

(Cytofluor 2300, Millipore, Bedford, MA) at 530 nm excitation and 590 nm emission wavelength. The decrease of fluorescence, as a measure for reduced trypanosome number, was calculated for each mAb by subtraction of the measured value from the background fluorescence. The average of the fluorescence units of the growth control without antibodies served as the 100% standard. The average fluorescence in the presence of a mAb minus average background fluorescence was expressed as percentage of the growth control.

***In vivo* parasite growth inhibition studies.** All animal studies were carried out based on a protocol approved by an animal ethics committee. *In vivo* activity of the anti-GPI mAbs was assessed in mice infected with either a GFP expressing *P. berghei* ANKA strain (1 times 10⁴ parasitized erythrocytes per mL)^[42] or with the *T. brucei rhodesiense* strain STIB 900 (1 times 10⁴ bloodstream form trypanosomes). Doses of 200µg antibodies formulated in PBS were administered intravenously 24 h before and 72 h after infection to six-week-old NMRI female mice obtained in sterile containers from RCC Ltd. (Füllinsdorf, Switzerland). Parasitemia levels were determined on several days after infection and the survival time in days was also recorded. For *T. brucei rhodesiense* tail blood smears were prepared and examined under a bright field microscope with a 200-fold magnification and a counting grid with defined dimensions. The degree of *P. berghei* ANKA infection (parasitaemia expressed as per cent infected erythrocytes) was determined by FACSscan flow cytometry (Becton Dickinson, San Jose, Calif.) according to.^[42]

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Figures

Fig. 1. Structures of the seven synthetic GPI-glycans (1-7) that were arrayed on a BSA-coated glass slide to build a GPI-glycan microarray. Attachment of GPI 6 to keyhole-limpet-hemocyanine (KLH) carrier protein afforded the conjugate used for immunization studies.

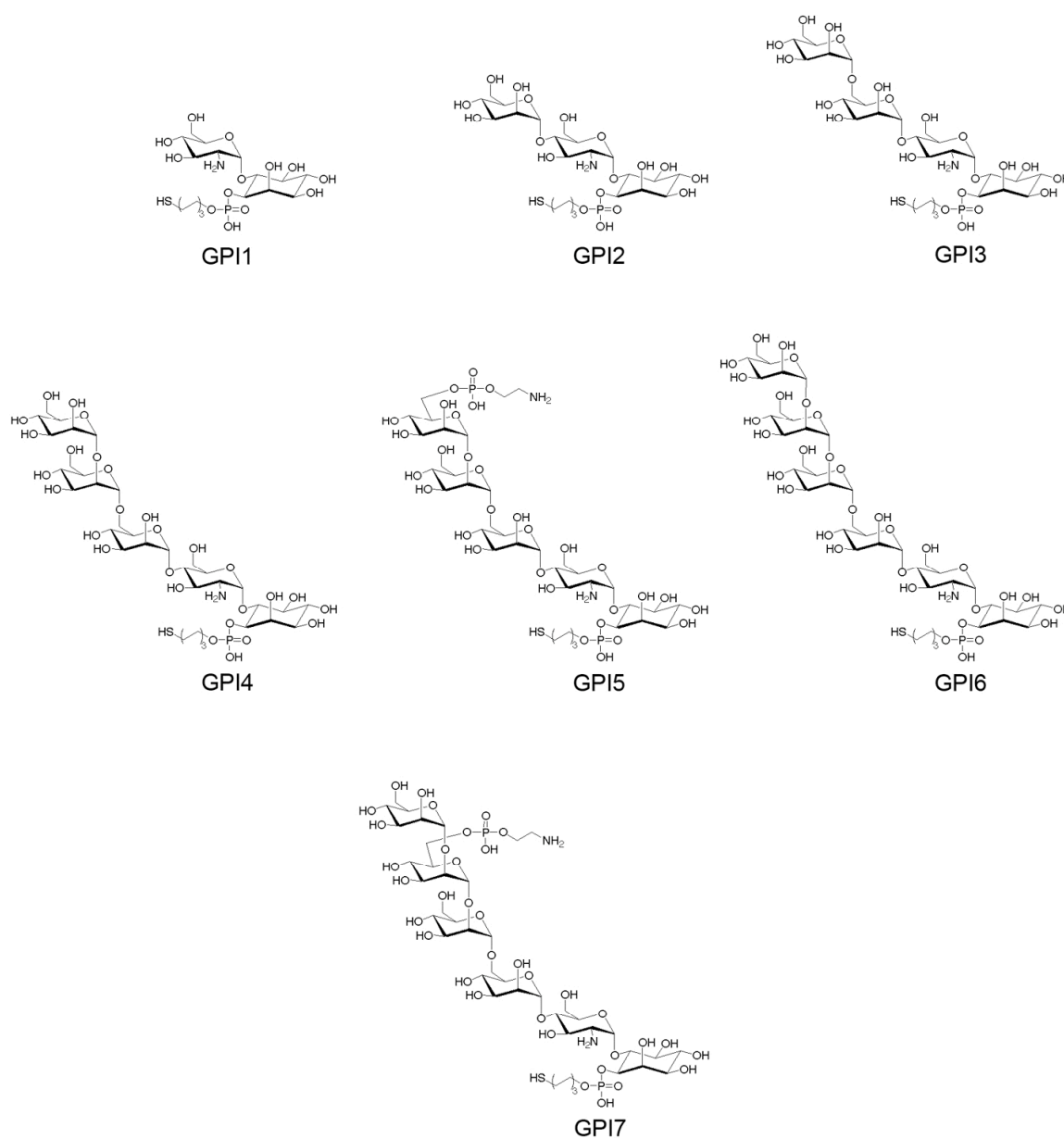


Fig. 2. A: Development of anti-GPI 1 to 7 IgG responses in mice after immunization with synthetic GPI 6 antigen conjugated to KLH formulated in ImmunEasy™ adjuvant. Sera were taken pre-immune, three weeks after the second and third immunization. Shown are readouts obtained with serial dilutions of sera of a representative mouse in synthetic *Pf*GPI microarray analysis. B: Fine specificities of the six generated anti-GPI mAbs. Serial dilutions of hybridoma supernatants were analysed in the microarray-based immunoassay. C: Prototype microarray images obtained after incubation with MTG1 and MTG6 mAbs. Serial four-fold dilutions of the hybridoma supernatants started at the upper right screening unit. D: Spotting order of the seven synthetic GPI-glycans (1-7) that were arrayed as quadruplicates in 64 identical screening units on a single slide.

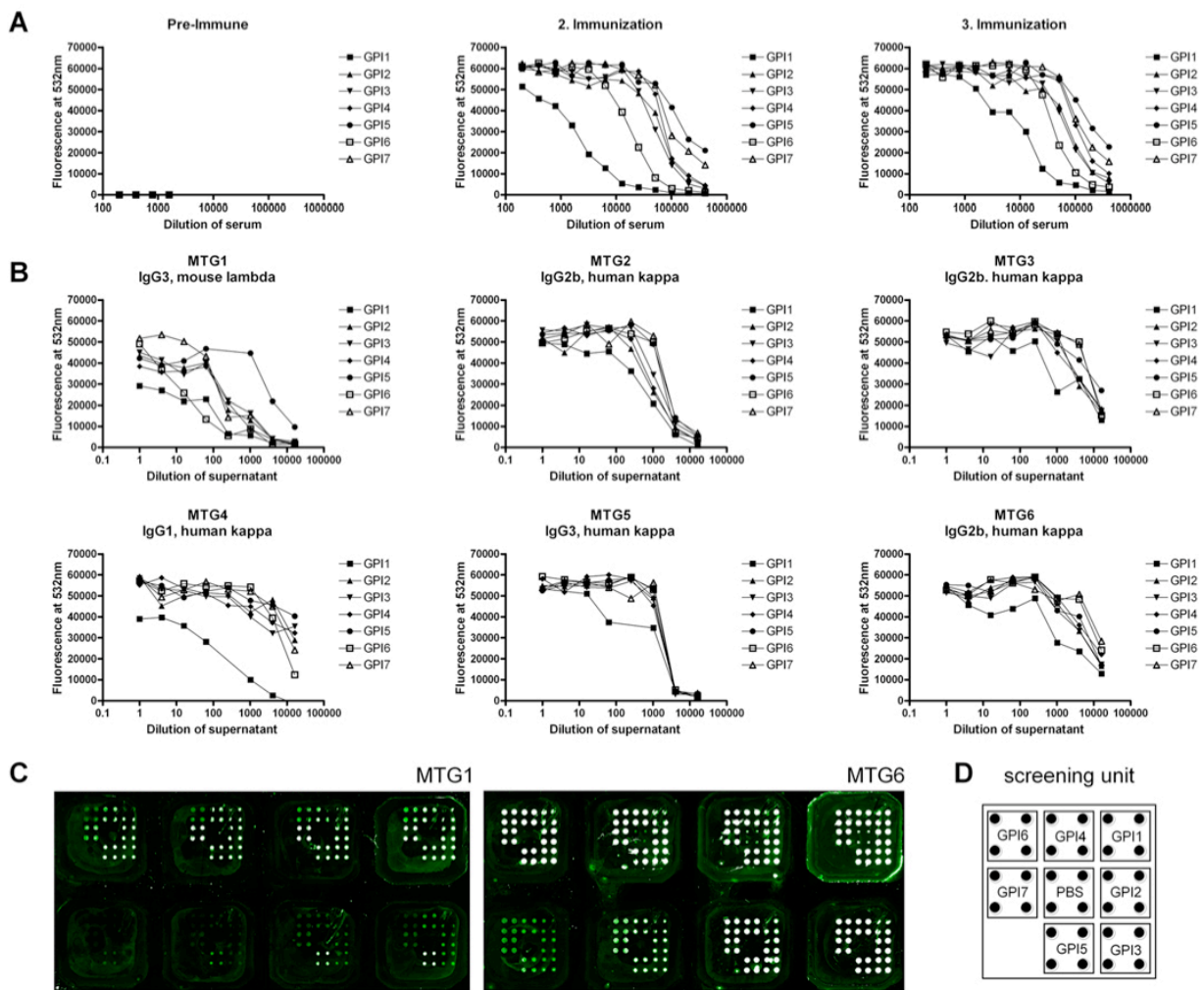


Fig. 3. Immunofluorescence staining of late trophozoites and schizonts of *P. falciparum* strain K1 and bloodstream-forms of *T. brucei rhodesiense* strain STIB 900. Parasites were stained with purified anti-synthetic GPI mAbs (4 μ g/ml) and a secondary antibody conjugated to Cy3. Nuclei were visualized using Hoechst dye no. 33256.

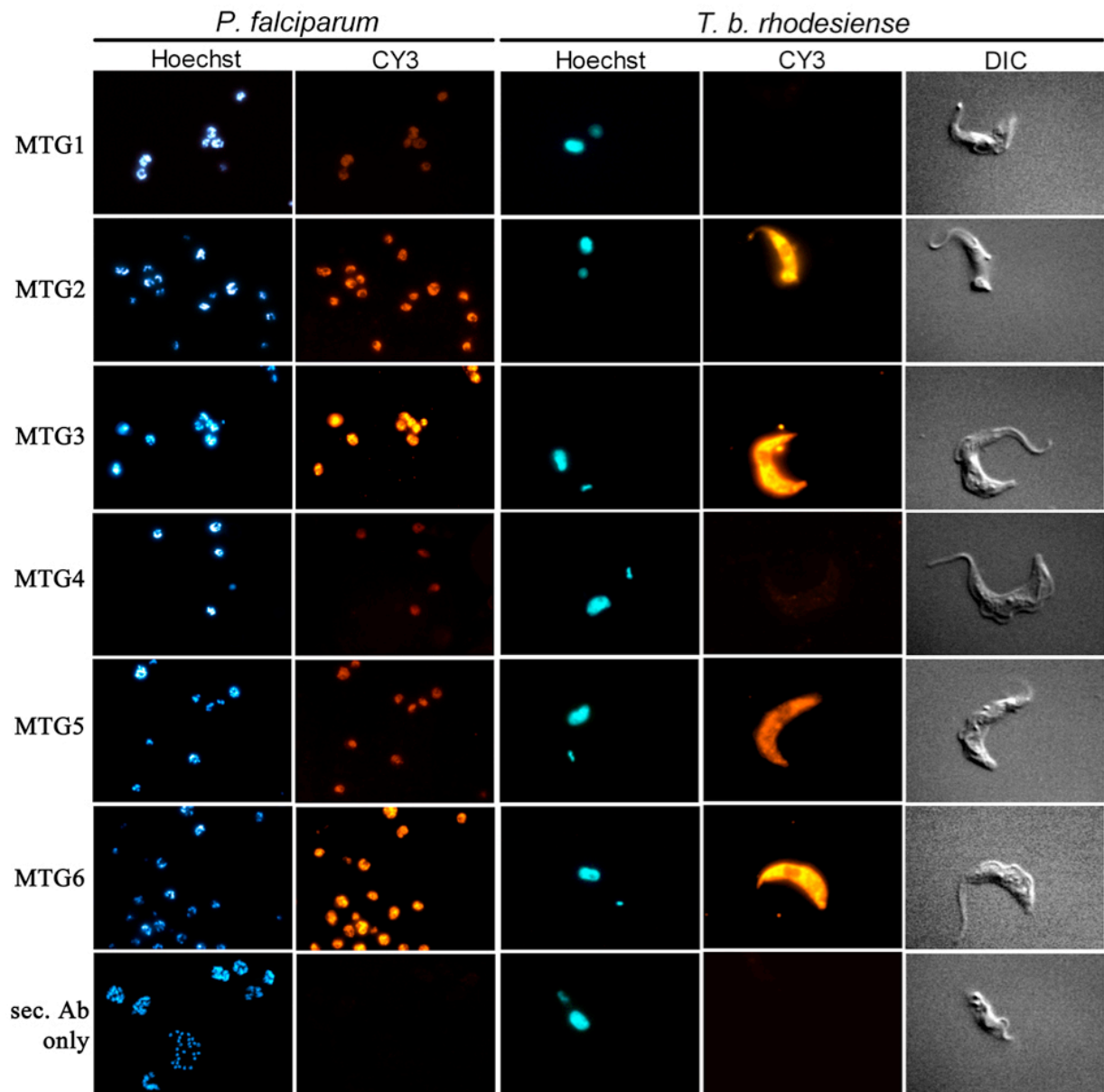


Fig. 4. A: Western blotting analysis with anti-synthetic GPI mAb MTG6 using *P. falciparum* blood stage lysates taken 6, 12, 18, 24, 30, 36, 42, and 48 h (lanes 1 to 8) after synchronization and salivary gland sporozoites (lane 9). An anti-GAPDH mAb served as a loading control. Western blotting analysis with mAb MTG6 with total cell lysates of asexual blood stages of *P. chabaudi* (B, lanes 1) and *P. berghei* (B, lanes 2) and of *in vitro* cultivated *T. brucei gambiense* (C, lane 1) and *T. brucei rhodesiense* (C, lane 2) lysates. Blots were developed using the ECL system. Comparable results were obtained with the two other Western blotting positive mAbs MTG2 and MTG3.

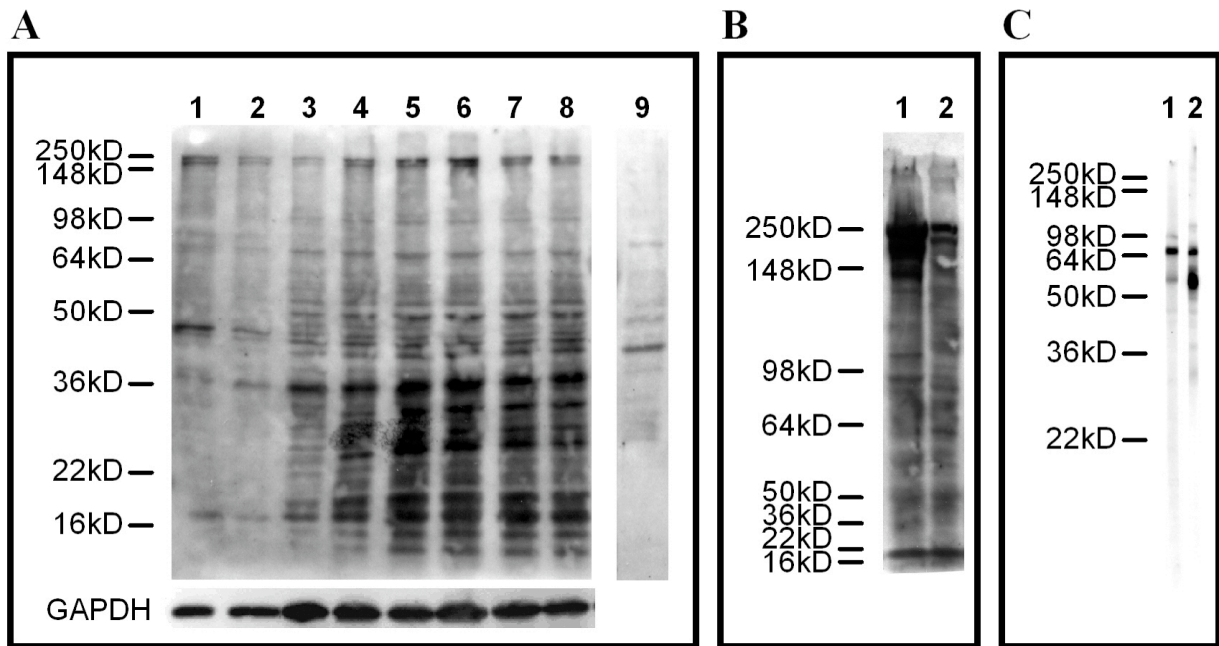
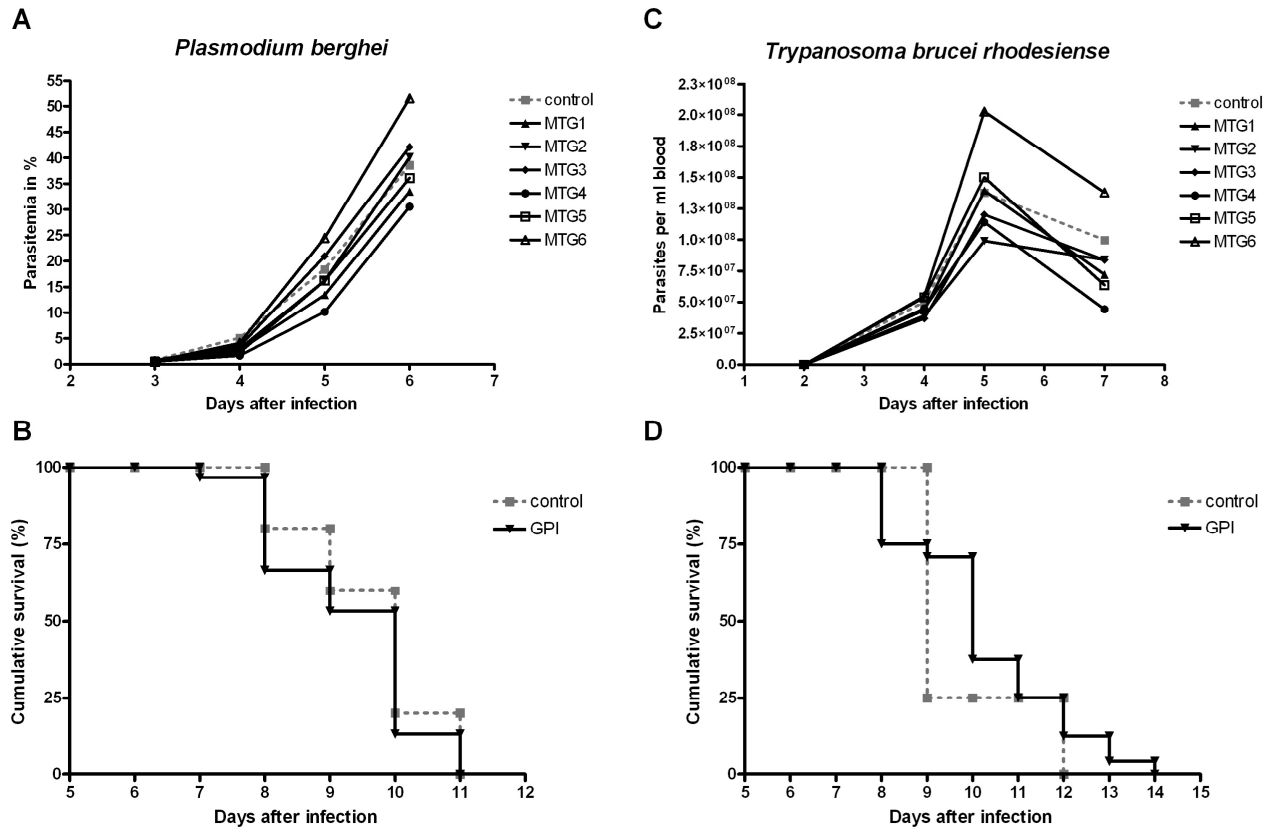
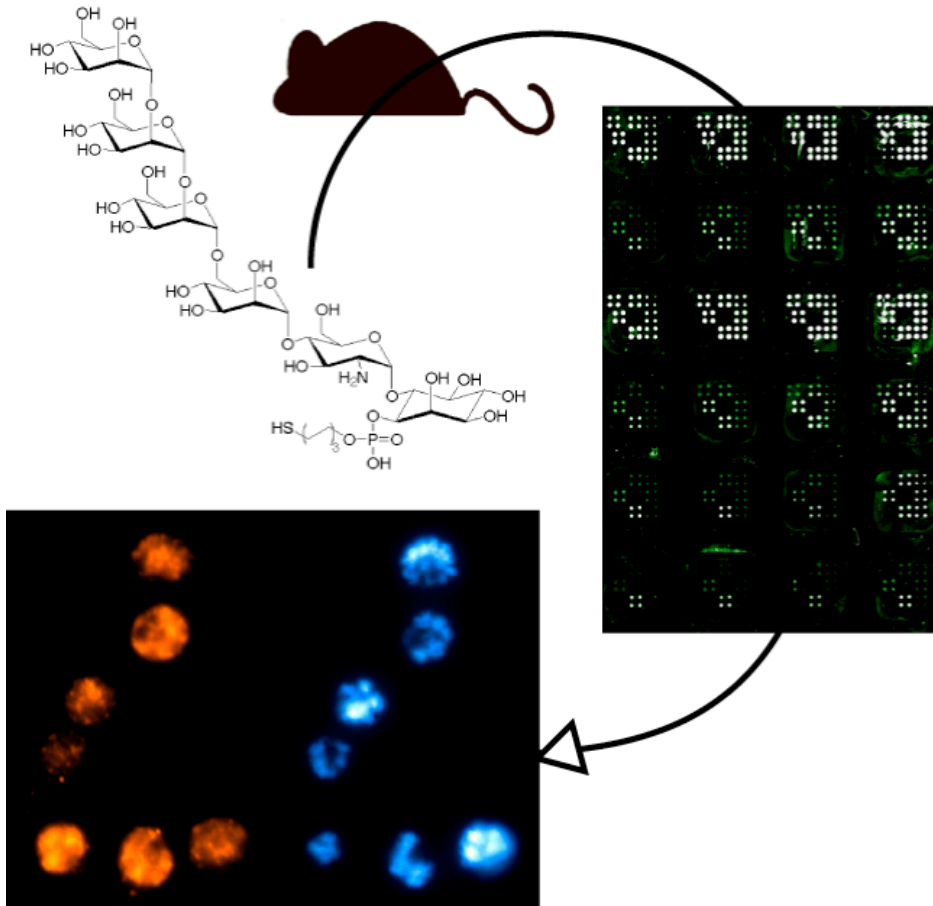


Fig. 5. Passive immunization studies with anti-synthetic GPI mAbs using murine infection models with either a GFP expressing *P. berghei* ANKA strain or with the *T. brucei rhodesiense* strain STIB 900. Parasitemia levels (A and C) were determined on several days after infection and the survival time in days was also recorded (B and D). Unvaccinated mice served as controls. Two further independent experiments with different batches of anti-GPI antibody preparations yielded comparable results.



Suggestion for the table of contents

Glycosylphosphatidylinositol (GPI) glycolipids of *Plasmodium falciparum* may be involved in the pathogenesis of malaria. GPIs are heterogeneous and difficult to isolate from their natural sources. Carbohydrate microarrays based on pure fully characterized synthetic GPI allowed the selection of GPI specific B-cell hybridomas and the first detailed description of the properties of anti-GPI monoclonal antibodies.



Keywords: GPI, malaria, microarrays, monoclonal antibodies, synthetic oligosaccharides

**CHAPTER 6: ANTI-CARBOHYDRATE ANTIBODIES FOR THE
DETECTION OF ANTHRAX SPORES**

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ANTI-CARBOHYDRATE ANTIBODIES FOR THE DETECTION OF ANTHRAX SPORES**

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Keywords

anthrax • carbohydrates • monoclonal antibodies • oligosaccharides • pathogen detection

Spores of *Bacillus anthracis* have been used as biowarfare agents to terrorize civilian populations. Once this durable form of the pathogen has been inhaled it will kill most victims if treatment does not commence within 24-48 h.^[1] After uptake into the body, the spores germinate and vegetative cells multiply and produce toxins, leading to the development of lesions from which the infection may disseminate, which is frequently fatal. It is assumed that an effective anthrax-subunit vaccine should contain multiple antigens. A surface antigen of the spores eliciting antibodies that suppress germination and enhance phagocytosis of spores by macrophages would be a suitable component for such a vaccine.

Several detection systems have been developed to identify *B. anthracis*^{[2]-[4]} and process the large number of samples that have to be tested each year. PCR-based assays or traditional phenotyping of bacteria are the most accurate but are also complex, expensive, and slow. Antibody binding to spore-surface peptide and protein antigens is less complicated, although it is plagued by higher false-positive and false-negative readouts. The similarity of spore-cell-surface antigens of the opportunistic human pathogen *B. cereus* and other bacteria of this group makes it difficult to create selective, reliable, antibody-based detection systems.

On the surface of *B. anthracis* spores, a unique tetrasaccharide **1** (Scheme 1) containing an entirely novel monosaccharide, named anthrose, was discovered recently.^[5] Access to usable quantities of pure cell surface oligosaccharides by isolation is often difficult and leaves synthetic chemistry as a last resort to procure carbohydrate antigens. We envisioned the detection of *B. anthracis* spores based on antibodies against carbohydrate antigen **1**, which is not present on related strains of bacteria.

The tetrasaccharide was chemically synthesized^{[6], [7]} in the form of the n-pentenyl glycoside **2**.^[6] The chemical handle on the reducing end was utilized to introduce a reactive terminal aldehyde moiety through ozonolysis. Covalent attachment^{[8], [9]} of synthetic **2** to the keyhole-limpet-hemocyanine (KLH) carrier protein by reductive amination produced a carbohydrate-protein conjugate **3** that was immunogenic in mice (Scheme 1). The conjugation to protein carriers is an efficient way to improve the immunogenicity of oligosaccharide antigens. The proteins provide peptide units that can be recognized by carrier-specific T cells, thus converting a T-cell-independent response against an unconjugated oligosaccharide into a more efficient T-cell-dependent response.

Tetrasaccharide-KLH conjugates formulated in ImmunEasy adjuvant (QIAGEN) elicited tetrasaccharide binding immunoglobulin G (IgG) antibodies after the third immunization in mice. Three B cell hybridoma lines producing tetrasaccharide specific monoclonal IgG antibodies (mAbs) were generated from spleen cells of one of the immunized mice. In an indirect immunofluorescence assay, all three mAbs bound specifically to native *B. anthracis* endospores. No binding was found

to endospores of *B. cereus*, *B. subtilis*, and *B. thuringiensis*, which are close relatives of *B. anthracis* (Figure 1).

These results demonstrate that distinct differences in cell-surface carbohydrate antigens can provide the basis for the generation of specific immunological reagents. This was demonstrated herein for the synthetic tetrasaccharide antigen **2** of *B. anthracis*. The tetrasaccharide-specific mAbs may be suitable to develop a highly sensitive and specific detection system for *B. anthracis* endospores and contribute to the development of novel therapeutic or preventive approaches.

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Figures

Scheme 1. Structure of the tetrasaccharide **1** found on the surface of *B. anthracis* spores and its synthetic analogue **2**. Attachment through a pentenyl handle to the KLH carrier protein afforded the conjugate **3**: a) O₃, MeOH, -78 °C, 10 min; b) Me₂S, MeOH, RT, 2 h; c) KLH carrier protein, PBS buffer solution (pH 7.2), NaBH₃CN, 37 °C, 48 h. PBS = phosphate-buffered saline.

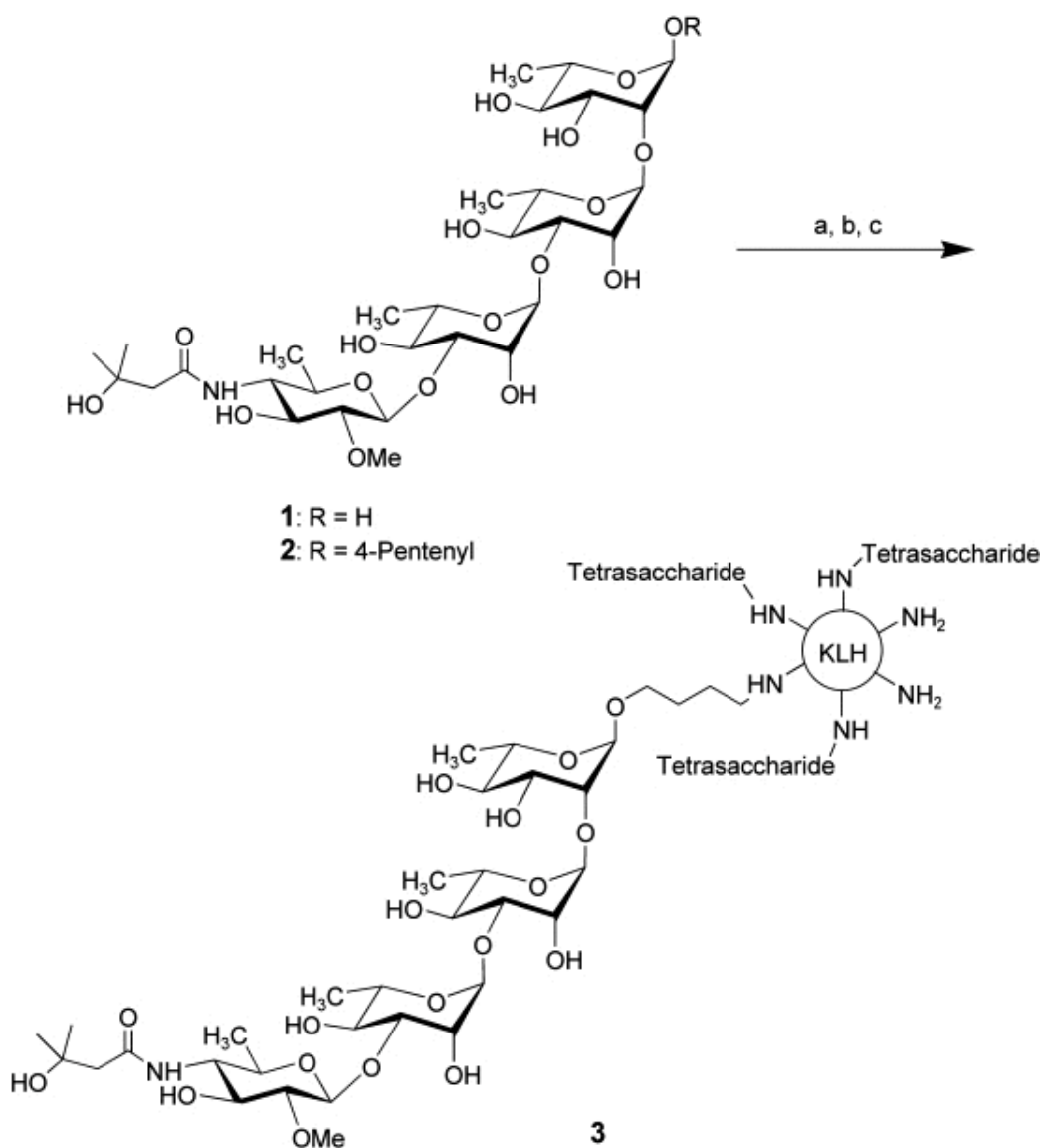
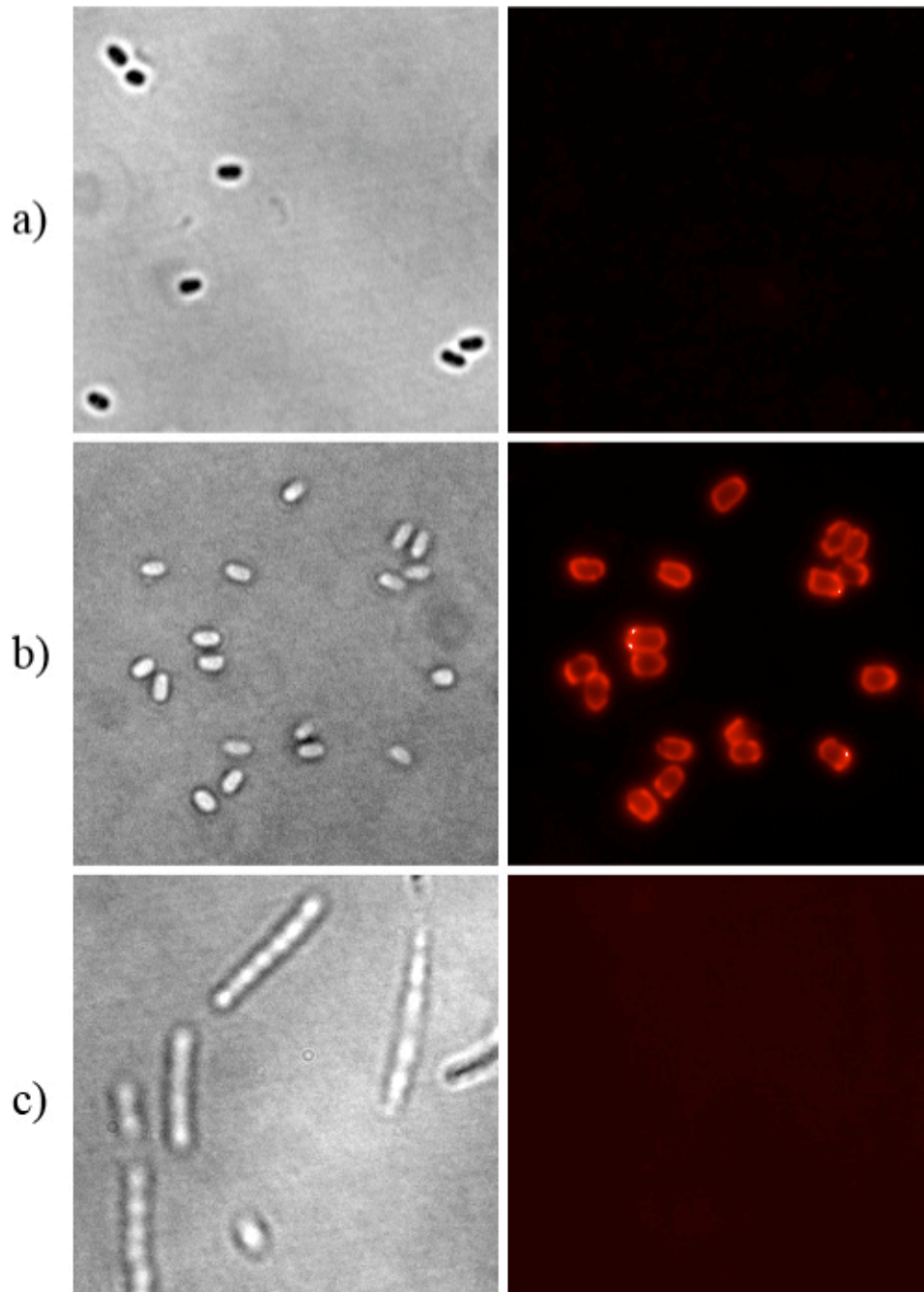
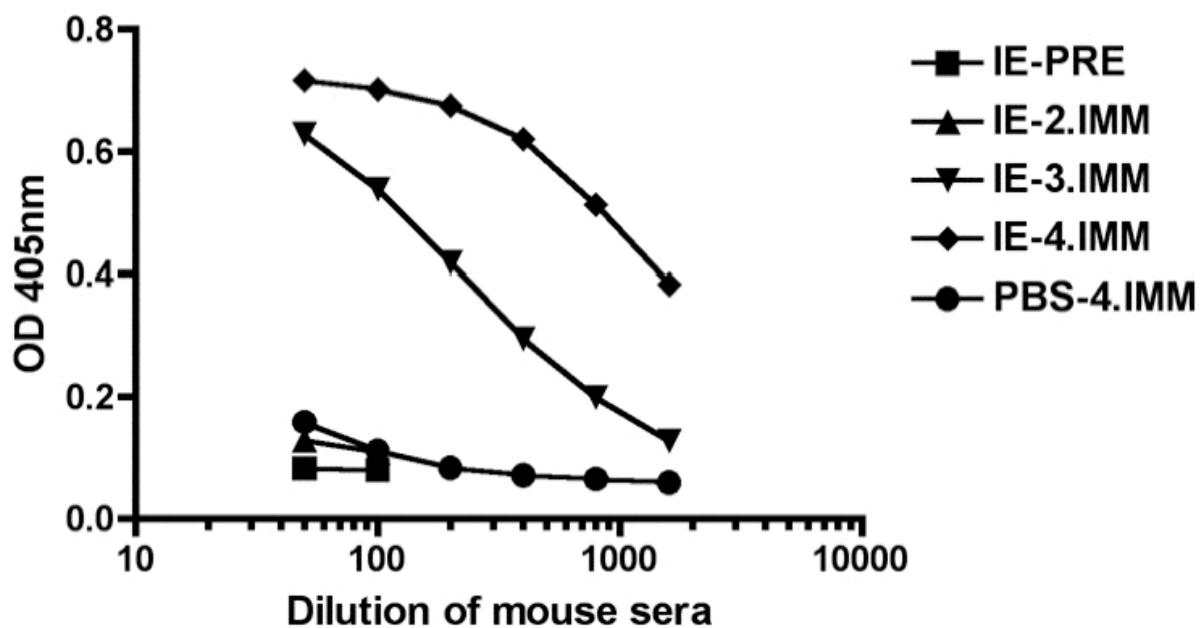


Figure 1. Indirect immunofluorescent staining of *B. anthracis* and *B. cereus* endospores by a mAb that was generated against the synthetic tetrasaccharide **2**. Left column: bright-field microscopy; right column: Cy3-specific immunofluorescence staining of a) *B. anthracis* spores with an isotype-matched control mAb, b) *B. anthracis* spores with a **2**-specific mAb, and c) *B. cereus* spores with a **2**-specific mAb.



Supporting Information

Figure SI 1. Tetrasaccharide specific IgG ELISA titers after second (2.IMM), third (3.IMM) and fourth (4.IMM) immunization of a mouse with KLH conjugate formulated in ImmunEasy™ (IE) or in phosphate buffered saline (PBS-4.IMM). Preimmune serum sample (PRE) showed no significant reactivity with the immunogen.



Ozonolysis of Tetrasaccharide 2 and Conjugation with Bovine Serum Albumine (BSA) and Keyhole Limpet Haemocyanin (KLH). Tetrasaccharide 2 (5 mg, 0.029 mmol) was dissolved in methanol (3 mL) and cooled to -78 °C. Ozone gas was passed through the solution for 10 min under vigorous stirring. A pale blue solution was formed. The excess ozone was displaced with nitrogen over a period of 10 min. The blue color disappeared. Methylsulfide (0.1 mL) was added and the reaction mixture was stirred for 2 h at room temperature. Afterwards, all the volatiles were removed under a stream of nitrogen. The resulting white solid was used directly in the subsequent conjugation step. MS (ESI) experiments showed the formation of the aldehyde. The solid was dissolved in 1 mL of 0.1 M phosphate buffered saline (PBS buffer), pH 7.2. The volume was divided into two times 0.5 mL, each of them in a separate flask. In one of them 4.2 mg of BSA was added, in the other 4.2 mg of KLH. To each of the two flasks 1 mg of sodium cyanoborohydride was added and the mixtures incubated under gentle agitation at 37 °C for 48 h. After 20 h, an additional 0.5 mg of sodium cyanoborohydride was added and the incubation continued. Both mixtures were diafiltered using a Amicon Centriprep with molecular weight cut-off value of 30 000 Da with six changes of PBS at 4 °C. Larger volumes (1.0 mL instead of 0.5 mL) of PBS buffer in the conjugation step resulted in a smaller number of tetrasaccharide moieties (three to four) attached to the protein. Due to the higher mass of KLH, we do not have comparative data for KLH.

Mouse Immunogenicity Studies. Mice carrying human immunoglobulin C γ 1 heavy and C κ light chain gene segments (Pluschke *et al.*, 1998) were immunized subcutaneously and intraperitoneal with tetrasaccharide–KLH conjugate formulated in ImmunEasyTM adjuvant (QIAGEN). Starting on day 0, they received at three-weekly intervals four doses of 20 μ g conjugate.

Generation of anti-Tetrasaccharide Monoclonal Antibodies (mAbs). Three days before cell fusion, a mouse immunized with the ImmunEasyTM formulation received an intravenous booster injection with 20 μ g of conjugate in phosphatebuffered saline. From the sacrificed mouse the spleen was aseptically removed and a spleen cell suspension in IMDM was mixed with PAI mouse myeloma cells as a fusion partner. Spleen and myeloma cells in a ratio of 1:1 were centrifuged; after the supernatant was discarded, the pellet was mixed with 1 mL pre-warmed polyethylene glycol 1500 sterile solution. After 60 s 10 mL of culture medium were added. After 10 min cells were suspended in IMDM containing hypoxanthine, aminopterin, thymidine, and 20% fetal bovine serum (HAT) and cultured in 96-well tissue culture plates. The cells were cultured at 37 °C with 5% CO₂ and fed 5 days later with 100 ml of HAT medium. Cells secreting tetrasaccharide-specific immunoglobulin (IgG) were identified by enzyme-linked immunosorbent assay (ELISA) using

Immunolon 4 plates (Dynerx Technologies Inc., Chantilly, Va.) coated with tetrasaccharide-BSA conjugate. Three hybridoma cell lines producing tetrasaccharide specific mAbs were identified and cloned twice by limiting dilution. Three mAbs, named MTA1, MTA2 and MTA3, were purified from spent culture supernatant of the three hybridoma clones by protein A affinity chromatography (HiTrap rProtein A FF, Amersham Biosciences). Purified MAbs were dialyzed against PBS with Slide-A-Lyzer Dialysis Cassette (PIERCE), aliquoted, and stored at -80 °C.

ELISA. ELISA microtiter plates Immunolon 4 (Dynerx Technologies Inc., Chantilly, Va.) were coated at 4 °C overnight with 50 µL of a 10 µg/mL solution of tetrasaccharide-BSA conjugate in PBS, pH 7.2. Wells were then blocked with 5% milk powder in PBS for 1 h at room temperature followed by three washings with PBS containing 0.05% Tween-20. Plates were then incubated with two-fold serial dilutions of mouse serum or hybridoma cell supernatants in PBS containing 0.05% Tween-20 and 0.5% milk powder for 2 h at room temperature. After washing, the plates were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (γ -chain specific) antibodies (Sigma, St. Louis, MO) for 1 h at room temperature and then washed. Phosphatase substrate (1 mg/mL *p*-nitrophenyl phosphate (Sigma)) in buffer (0.14% Na₂CO₃, 0.3% NaHCO₃, 0.02% MgCl₂, pH 9.6) was added and incubated at room temperature. The optical density (OD) of the reaction product was recorded after appropriate time at 405 nm using a microplate reader (Titertek Multiscan MCC/340, Labsystems, Finland).

MAb Isotyping. The isotypes of the generated mAbs were determined by ELISA. ELISA microtiter plates Immunolon 4 (Dynerx Technologies Inc., Chantilly, Va.) were coated at 4 °C overnight with 50 µL of a 10 µg/mL solution of tetrasaccharide-BSA conjugate in PBS buffer. Wells were then blocked with 5% milk powder in PBS followed by three washings with PBS containing 0.05% Tween-20; then 100 µL of tested supernatants were added to different wells, which were incubated and washed again. Plates were incubated for 1 h with alkaline phosphatase-conjugated goat anti-mouse antibodies specific for IgG (Sigma); IgG1; IgG2b; IgG3; IgM; (Southern Biotech) and with alkaline phosphatase-conjugated mouse anti-human antibodies specific for IgG1 and κ (Southern Biotech). After washing, phosphatase substrate was added and incubated at room temperature. The optical density of the reaction product was recorded at 405 nm.

Immunofluorescence Assays (IFA) for Detection of *Bacillus anthracis* endospores. Endospore suspensions were added to microscope glass slides, air-dried and fixed twice with acetone. Slides were incubated in a moist chamber for 30 min at room temperature with either mouse serum diluted in PBS, hybridoma supernatants or purified mAbs diluted in PBS. After five

washes with PBS, Cy3-conjugated affinity-pure F(ab')₂ fragment goat anti-mouse IgG antibodies (Jackson Immuno Research Laboratories, West Grove, PA), were added to the slides and incubated for 30 min at room temperature. Afterwards, slides were washed five times with PBS and covered with a cover slip. Antibody binding was assessed by fluorescence microscopy. The *B. anthracis* strains used for IFA were Pasteur 1, A73202 and Sterne, NCTC8234 and the *B. cereus* strain was JF1887 (ATCC9634).

**CHAPTER 7: IMMUNO-DETECTION OF ANTHROSE
CONTAINING TETRASACCHARIDE IN THE
EXOSPORIUM OF *BACILLUS ANTHRACIS* AND
BACILLUS CEREUS STRAINS**

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**IMMUNO-DETECTION OF ANTHROSE CONTAINING
TETRASACCHARIDE IN THE EXOSPORIUM OF *BACILLUS
ANTHRACIS* AND *BACILLUS CEREUS* STRAINS**

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Running Title

Immuno-detection of anthrose in the *B. cereus* group

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Abstract

Aims: *Bacillus anthracis* strains of various origins were analysed with the view to describe intrinsic and persistent structural components of the *Bacillus* collagen-like protein of anthracis glycoprotein associated anthrose containing tetrasaccharide in the exosporium.

Methods and Results: The tetrasaccharide consists of three rhamnose residues and an unique monosaccharide – anthrose. As anthrose was not found in spores of related strains of bacteria, we envisioned the detection of *B. anthracis* spores based on antibodies against anthrose-containing polysaccharides. Carbohydrate–protein conjugates containing the synthetic tetrasaccharide, an anthrose–rhamnose disaccharide or anthrose alone were employed to immunize mice. All three formulations were immunogenic and elicited IgG responses with different fine specificities. All sera and monoclonal antibodies derived from tetrasaccharide immunized mice cross-reacted not only with spore lysates of a panel of virulent *B. anthracis* strains, but also with some of the *B. cereus* strains tested.

Conclusions: Our results demonstrate that antibodies to synthetic carbohydrates are useful tools for epitope analyses of complex carbohydrate antigens and for the detection of particular target structures in biological specimens.

Significance and Impact of the Study: Although not strictly specific for *B. anthracis* spores, antibodies against the tetrasaccharide may have potential as immuno-capturing components for a highly sensitive spore detection system.

Introduction

Anthrax is an acute zoonotic disease caused by the spore-forming, rod-shaped bacterium *Bacillus anthracis* that can lie dormant in the soil for decades. Upon ingestion by grazing animals, the spores germinate, changing from the resistant form into the growing and toxin producing vegetative form. After the bacteria disseminate, they typically kill the infected animal and return to the environment converting again to spores. *B. anthracis* has been described as the ultimate biological weapon because of its virulence and persistence when disseminated as spores (Inglesby *et al.* 1999, Borio *et al.* 2001).

Human vaccines for anthrax are available (Turnbull 2000, Pittman *et al.* 2001) but there has been much controversy over the safety and effectiveness of the current vaccines. Research on a second-generation vaccine in recent years was based on the observation that antibodies to protective antigen (PA) are crucial for protection against exposure to virulent anthrax spores (Brey 2005). Since antibodies to PA address the toxemia component of anthrax disease, it is assumed that an effective anthrax-subunit vaccine should contain multiple antigens. Inclusion of killed *B. anthracis* spores enhances the protective efficacy of PA-based vaccines in animal models (Brossier 2002). In order to generate an immunity that protects from infection with *B. anthracis*, the capsule or somatic antigens in the spore may represent critical vaccine components.

The exosporium, the primary permeability barrier of the spore and the source of spore surface antigens has been the focus of recent investigations. As the outermost surface of the spore, the exosporium is likely to be the most immunological accessible structure of the spore. The glycoprotein Bacillus collagen-like protein of anthracis (BclA) was the first exosporium protein that was identified and has been shown to be an immunodominant protein suggesting a role for this protein in spore-host interactions (Steichen *et al.* 2003). BclA has conserved amino- and carboxy-termini and a long, central collagen-like region that is similar to mammalian collagen proteins (Sylvestre *et al.* 2002, Sylvestre *et al.* 2003). This polymorphic collagen-like region consists of GXX repeats, including a large proportion of GPT triplets, and (GPT)₅GDTGTT repeats. The latter 21-amino-acid repeat has been named BclA repeat (Sylvestre *et al.* 2002) and appears to be an essential feature of the BclA protein important for the structural organization of BclA on the spore surface. The number of GXX repeats in the collagen-like region varies among strains (Steichen *et al.* 2003, Sylvestre *et al.* 2003) and is responsible for the different lengths of the exosporium filament found on spores of different *B. anthracis* strains (Sylvestre *et al.* 2003). Two *O*-linked carbohydrates attached to BclA, a 715-Da tetrasaccharide and a 324-Da disaccharide, have been identified (Daubenspeck *et al.* 2004). Multiple copies of the tetrasaccharide are linked to the collagen-like region of BclA, whereas the disaccharide may be attached outside of this region. Most

of the collagen-like region repeating units contain a threonine residue that provides sites for potential glycosylation via a GalNAc linker. The tetrasaccharide is composed of three rhamnose residues and anthrose, an unusual sugar that was not found in spores of strains belonging to the phylogenetically most similar species *B. cereus* and a *B. thuringiensis* (Daubenspeck *et al.* 2004, Tamborrini *et al.* 2006). Here, we demonstrate cross-reactivity of antisera and monoclonal antibodies raised against the synthetic *B. anthracis* tetrasaccharide and truncated anthrose containing structures using immunoblots with lysed spores of *B. anthracis* and *B. cereus* strains.

Material and Methods

Synthesis of carbohydrates. The tetrasaccharide **I** was prepared as described before by our group (Werz and Seeberger 2005, Werz *et al.* 2007). Following our initial report, four other groups achieved the synthesis of this tetrasaccharide or corresponding sequences (Adamo *et al.* 2005, Saksena *et al.* 2005, Mehta *et al.* 2006, Crich and Vinogradova 2007, Guo and O'Doherty 2007, Saksena *et al.* 2006, Saksena *et al.* 2007). The disaccharide **II** as well as the anthrose **III** used for further vaccination studies were prepared in an analogous manner by our modular approach (Werz and Seeberger 2005, Werz *et al.* 2007). The experimental details for the preparation of **II** and **III** as well as their analytical data are described in the supplementary material (Figs S1 and S2). The terminal double bonds of **II** and **III** were transformed into thiol functionalities suitable for reaction with a maleimide-functionalized KLH carrier protein. These KLH-glycoconjugates were used for the mouse immunogenicity studies.

Bacterial strains. *B. anthracis* strains selected from different genotypes (Maho *et al.* 2006, Pilo *et al.* 2008) are described in Table 1. *B. thuringiensis* ATCC 29730; *B. cereus* ATCC 10876; *B. cereus* ATCC 10876; *B. cereus* ATCC 13061; *B. cereus* ATCC 14579; *B. cereus* ATCC 33019, were purchased from the American Type Culture Collection (Rockville, MD, USA.). *B. cereus* F4370/75 (Cereus III lineage, ST-27); *B. cereus* m1545 (Cereus I lineage; ST-5); *B. cereus* m1564 (Cereus I lineage; ST-6); *B. cereus* m1293 (Cereus II lineage; ST-45); *B. cereus* F4810/72 (Cereus II lineage; ST-26) were obtained from F. G. Priest (Priest *et al.* 2004).

Production of spores from *Bacillus anthracis*. Strains of *Bacillus anthracis* were cultured on tryptone soya agar with 5% sheep blood (Oxoid, Basel, Switzerland) at 37°C for 18 h. Then the culture plates were kept at room temperature for 4 weeks until the colonies appeared dry. Subsequently colonies were suspended in 1 ml PBS buffer (50 mM Na₂HPO₄ - NaH₂PO₄ pH 8.0, 140 mM NaCl) per culture plate, heated at 80°C for 10 min. Spores were then collected by centrifugation at 4000 x g for 15 min and suspended in PBS at a titer of 10⁹ spores per ml.

Mouse immunogenicity studies. Mice carrying human immunoglobulin C γ 1 heavy and C κ light chain gene segments (Pluschke *et al.* 1998) were immunized with synthetic saccharide antigens (Fig 1.) conjugated to keyhole-limpet-hemocyanine (KLH) formulated in ImmunEasyTM adjuvant (QIAGEN). Starting on day 0, they received at three-weekly intervals three doses of 40 μ g conjugate. Blood was collected before each immunization and 2 weeks after the final injection. The production of anti-tetrasaccharide monoclonal antibodies is described in (Tamborrini *et al.* 2006).

Anti- *B. thuringiensis* antiserum was generated by immunization with strain ATCC 29730 *B. thuringiensis* spores (Raven Biological Laboratories, INC, Omaha, NE). Mice were immunized subcutaneously at three-weekly intervals with 10^6 *B. thuringiensis* spores formulated in 0.2 ml PBS without adjuvant. Two weeks after the third injection, heart blood was collected and serum stored at -20° C until use.

Animals were housed in temperature-controlled rooms (22° C, $\pm 3^{\circ}$). Conventional laboratory feeding and unlimited drinking water were provided to the mice. Approval for animal experimentation has been obtained from the responsible authorities and all experiments have been performed in strict accordance with the Rules and Regulations for the Protection of Animal Rights laid down by the Swiss Bundesamt für Veterinärwesen. All animal manipulations have been performed under controlled laboratory conditions by specifically qualified personnel in full conformity to Swiss and European regulations.

Enzyme-linked immunosorbent assay (ELISA). ELISA microtiter plates Immunolon 4 (Dynex Technologies Inc., Chantilly, Va.) were coated at 4° C overnight with $50\mu\text{l}$ of a $10\mu\text{g ml}^{-1}$ solution of saccharide-BSA conjugate in PBS, pH 7.2. Wells were then blocked with 5% milk powder in PBS for 1 h at room temperature followed by three washings with PBS containing 0.05% Tween-20. Plates were then incubated with two-fold serial dilutions of mouse serum or mAbs in PBS containing 0.05% Tween-20 and 0.5% milk powder for 2 h at room temperature. After washing, the plates were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (γ -chain specific) antibodies (Sigma, St. Louis, MO) for 1 h at room temperature and then washed. Phosphatase substrate (1 mg ml^{-1} *p*-nitrophenyl phosphate (Sigma)) in buffer (0.14% Na_2CO_3 , 0.3% NaHCO_3 , 0.02% MgCl_2 , pH 9.6) was added and incubated at room temperature. The optical density (OD) of the reaction product was recorded after appropriate time at 405 nm using a microplate reader (Titertek Multiscan MCC/340, Labsystems, Finland).

PCR and DNA sequencing. PCR was performed using FIREPol® (Solis BioDyne, Tartu, Estonia) Taq polymerase and synthetic oligonucleotides hybridizing to flanking or internal sequences of the *bclA* gene. A segment including the entire *bclA* gene was amplified by using the primers $5'\text{-CCGTTAGAATCCATTGCAAGATGATAAGGC-3}'$ and $5'\text{-CGACCAACCATACTGTGTGCAGCTCTTGGC-3}'$ (Sylvestre *et al.* 2003). The sequence encoding the BclA variable collagen-like region containing the GXX-repeats was amplified by using the primers $5'\text{-CCCTAATCTTGTAGGACCTACATTACCACC-3}'$ and $5'\text{-CCCACCGGAGTTAAATGCATATAGTCCTGC-3}'$ (Sylvestre *et al.* 2003). $30\mu\text{l}$ reactions were carried out with a GeneAmp® 9700 PCR System (Applied Biosystems). Template DNA was

denatured at 94 °C for 4 min. Thirty amplification cycles of 30 s of denaturation at 94 °C, 30 s of annealing at 58 °C, and 2 min of extension at 72 °C were performed followed by one cycle of 10 min at 72 °C. The PCR products were analyzed by agarose gel electrophoresis and sequenced by using two primers flanking the *bclA* gene (5'-CGTGTCATTTTCTTTTCGGTTTTGCATCTAC-3' and 5'-GTGCCTCCTACGGAATGTCATAACAAC-3') by MACROGEN (Korea).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Endospore suspensions were solubilized with 2x loading buffer (1.7 ml of 0.5 M Tris-HCl [pH 6.8], 2 ml of glycerol, 4.5 ml of 10% sodium dodecyl sulfate, 1 ml of β -mercaptoethanol, 0.8 ml of bromophenol blue [0.3%, wt/vol]) and heated 10 min at 95 °C before loading on the 10 % SDS-PAGE. As molecular weight marker SeeBlue®Plus (Invitrogen) was used. Separated proteins were electrophoretically transferred to a nitrocellulose filter (Protran Nitrocellulose, BA85; Schleicher & Schuell) by semidry blotting. Blots were blocked with PBS containing 5% milk powder and 0.1% Tween 20 overnight at 4 °C. Whole blots or cut strips were incubated with appropriate dilutions of immune serum or mAbs in blocking buffer for 2 h at room temperature. In competition experiments primary antibodies were pre-incubated for 30 min with synthetic competitors. After several washing steps, blots were incubated with goat anti-mouse IgG horseradish peroxidase conjugated Ig (BioRad Laboratories, CA, USA) or with alkaline phosphatase-conjugated goat anti-mouse {gamma} heavy-chain antibodies (Sigma, St. Louis, Mo.) for 1 h. Blots were finally developed either using the ECL system according to manufacturer's instructions or with 5-bromo-4-chloro-3-indolylphosphate (BioRad, Reinach, Switzerland) and nitroblue tetrazolium (BioRad) to visualize bands.

Results

Fine specificity of antibodies raised against anthrose-containing synthetic carbohydrates. Synthetic *B. anthracis* tetrasaccharide, a rhamnose-anthrose disaccharide and anthrose (Fig. 1) were covalently attached to the keyhole-limpet-hemocyanine (KLH) carrier protein by reductive amination to improve their immunogenicity by the recruitment of carrier-specific T cells. Mice were repeatedly immunized with a CpG based adjuvant (ImmunEasy™, QIAGEN) formulation of the conjugates and the fine specificity of the immune sera was analyzed by ELISA and immunoblotting.

All anthrose monosaccharide-immunized animals developed high IgG ELISA titers to anthrose. Cross-reactivity with the disaccharide and the tetrasaccharide antigen was comparatively low (Fig. 2A). Sera from anthrose-rhamnose disaccharide immunized mice reacted best with the disaccharide immunogen itself, but also showed cross-reactivity with the anthrose monosaccharide and the *B. anthracis* tetrasaccharide (Fig. 2B). Sera and mAbs from tetrasaccharide immunized mice were highly specific for the tetrasaccharide (Fig. 2C and D, respectively). The three tetrasaccharide specific mAbs that were generated previously (Tamborrini *et al.* 2006) shared the IgG2b:λ isotype and exhibited similar fine specificity in all analyses.

Cross-reactivity of sera raised against the synthetic carbohydrate structures with the tetrasaccharide expressed by *B. anthracis* on the BclA protein was investigated by immunoblotting after separation of *B. anthracis* endospore lysate proteins by SDS-PAGE. With the endospore lysate of strain 1 (Table 1), all sera stained a double band with an apparent molecular weight of around ~250 kDa, corresponding to the BclA glycoprotein (Fig. 3). In competition experiments the binding of the anti-tetrasaccharide mAbs (Fig. 3C) and of anti-tetrasaccharide serum IgG (data not shown) to the spore antigen of *B. anthracis* strain 1 was inhibited only by the synthetic tetrasaccharide in a concentration dependent manner. Partial structures, such as trirhamnose, rhamnose-anthrose disaccharide, monorhamnose (data not shown), anthrose and a variant of anthrose (data not shown) missing the 3-hydroxy-3-methylbutamido side chain (Fig. 3D) failed to compete the strong binding to the native tetrasaccharide antigen. Antigen binding of anti-disaccharide antiserum (Fig. 3B) was blocked by tetrasaccharide and disaccharide, but not by anthrose. All competitors containing anthrose blocked antigen binding of anti-anthrose immune serum, whereas the side chain variant of anthrose did not inhibit binding (Fig. 3C).

Diversity of post-translational modification of the BclA protein in different *B. anthracis* strains. Immunoblotting experiments with spore lysates of a set of 11 *B. anthracis* isolates (Table 1) yielded strain-specific multiple band staining patterns characteristic for the BclA

protein (Sylvestre *et al.* 2003), when an anti-*B. thuringiensis* antiserum was used as primary antibody (Fig. 4E). Although the BclA proteins have a calculated molecular-weight of only 20 – 39 kDa, both high molecular weight bands (>150kDa) and low molecular weight bands (~50kDa) appear, due to glycosylation of the protein (Sylvestre *et al.* 2003). Patterns obtained with the tetrasaccharide specific mAbs (Fig. 4B) and immune sera (data not shown) included high-molecular-weight (>150kDa) bands for all strains tested. In the case of strains 1 and 2 as well as the „Sterne“ (34F2) strain (data not shown), staining of the high molecular weight band was very intense and no other bands became visible. Intense staining of low molecular weight bands was observed with strains 3 and 4 (~55kDa) and 5 to 8 (~50kDa) but not with strains 9 to 11, indicating a possible difference between strains in the levels of glycosylation. With disaccharide (Fig. 4C) and anthrose (Fig. 4D) specific immune sera comparable staining patterns were observed. However, with strains 3 to 11 staining of the high-molecular-mass bands in relation to the low molecular mass bands was increased, indicating that accessibility of the different epitopes differs for the high and low molecular weight bands.

To test whether immunoblot patterns correlated with the size and structure of the tetrasaccharide-carrying collagen-like region of the BclA protein, the *bclA* genes of the 11 *B. anthracis* strains were amplified by PCR and sequenced. While the amino- and carboxy-terminal sequence stretches were conserved, a wide variation in size of the central collagen-like regions from 69 to 219 amino acids was observed (Table 1), as reflected by size differences of the PCR products (Fig 4A). The collagen-like regions varied in the numbers of GXX units and the number of BclA repeats, defined by (Sylvestre *et al.* 2003) as the 21-amino-acid sequence (GPT)₅GDTGTT. In all eleven *B. anthracis* strains the same sets of four nucleotide sequences coded for the GPT repeats and the BclA repeats, respectively (data not shown).

Strains 1 and 2 associated with strong staining of the high-molecular-weight (>150kDa) bands had much larger collagen-like regions than the other strains (Table 1). The collagen-like regions of strains 3 and 4 that exhibited an additional ~55kDa molecular weight band in immunoblotting were slightly larger than those of strains 5 to 8 that showed a slightly smaller (~50kDa) band. The *bclA* genes of strains 9 to 11 that exhibited no low molecular weight bands were identical to those of strains 5 to 8. As indicated by gene exchange experiments (Sylvestre *et al.* 2003), variations in the BclA protein banding patterns thus can be caused by posttranslational modifications that depend more on the genetic background of the strains than on the sequences of the *bclA* genes.

Cross-reactivity of antisera with endospores of *B. cereus*. In immunoblotting experiments with spore lysates of nine *B. cereus* strains covering the Cereus I, II and III lineages from the MLST

B. cereus clade 1 (Priest *et al.* 2004), tetrasaccharide specific mAbs (Fig. 5) as well as tetra-, di- and monosaccharide specific immune sera (data not shown) exhibited reactivity with three strains. Staining of a high-molecular-weight band with an anti-*B. thuringiensis* spore antiserum demonstrated the expression of the immunodominant BclA protein in all *B. cereus* strains.

Discussion

Specific detection and phenotypic differentiation of *B. anthracis* is challenging (Williams *et al.* 2003), due to its genetic similarity to other bacteria of the *B. cereus* group (Helgason *et al.* 2000). Extensive genomic studies on strains of *B. anthracis*, *B. cereus* and *B. thuringiensis* have suggested that *B. anthracis* and *B. thuringiensis* are sub-species of the species *B. cereus*. The main difference between these sub-species is the presence of plasmids coding for insecticidal toxins in *B. thuringiensis* and the presence of the capsule plasmid pXO2 and the toxin plasmid pXO1 in *B. anthracis* (Edwards *et al.* 2005). DNA homology studies of different *B. anthracis* strains revealed evanescent small amounts of genomic variation. This significant homogeneity of *B. anthracis* may be the result of the organism surviving the majority of its life as a spore, where it is not exposed to DNA-altering events (Henderson *et al.* 1995, Helgason *et al.* 2000, Schupp *et al.* 2000). The BclA protein is an immunodominant spore antigen and it has been shown that the BclA protein itself and not its carbohydrate constituent directs the dominant immune response (Steichen *et al.* 2003). Identification of a BclA protein-associated tetrasaccharide with a previously undescribed terminal sugar anthrose in the animal vaccine strain “Sterne” of *B. anthracis* (Daubenspeck *et al.* 2004) had raised hope to have identified a *B. anthracis* specific antigen on the surface of the spore. It was proposed to use this antigen to develop a specific oligosaccharide-based detection system for *B. anthracis* spores, since anthrose was not found in spores of the *B. cereus* T strain and a *B. thuringiensis* ssp. *kurstaki* strain (Daubenspeck *et al.* 2004). The structural analysis of the tetrasaccharide was confirmed by our finding that monoclonal antibodies raised against the synthetic tetrasaccharide are cross-reactive with intact *B. anthracis* endospores of *B. anthracis* avirulent strain “Sterne” and highly virulent strain Ames in immunofluorescence assay (Tamborrini *et al.* 2006). Here we have tested these monoclonal antibodies and antisera raised against the tetrasaccharide, a rhamnose-anthrose disaccharide and anthrose for cross-reactivity with immunoblotted spore lysates of panels of *B. anthracis* and *B. cereus* strains. In immunoblotting experiments mAbs and immune sera cross-reacted with all *B. anthracis* isolates, but also with some of the *B. cereus* strains tested. The observed cross-reactivity demonstrates a broader presence of anthrose in the *B. cereus* group, supporting the phenotypic argument that *B. cereus*, *B. thuringiensis* and *B. anthracis* could be considered as a single bacterial species (Helgason *et al.* 2000). Recent genetic evidence demonstrating the presence of the anthrose biosynthetic operon in some *B. cereus* strains supports our findings (Dong *et al.* 2008). The collagen like region of the *B. cereus* BclA proteins contains GXX units harboring threonine residues representing sites for attachment of *O*-linked oligosaccharides (Castanha *et al.* 2006).

Four different BclA-related multiple-band patterns were observed. The multiplicity of bands is also seen when *B. anthracis* lysates are analyzed with anti-BclA protein antibodies (Sylvestre *et al.* 2003). We investigated whether the size and structure of the *bclA* genes and the corresponding proteins explains the observed differences in the anti-carbohydrate immunoblot patterns. Sequence differences were restricted to the polymorphic collagen-like regions of the BclA protein and correlations between the size of the collagen-like regions and the relative apparent molecular weights on SDS-PAGE gels were observed. However, for the *B. anthracis* strains 5 to 11, two different anti-tetrasaccharide blotting patterns were observed, while their *bclA* genes are identical. It has been demonstrated by gene exchange experiments (Sylvestre *et al.* 2003), that genetic background dependent posttranslational modifications of the BclA protein can cause such variations in the banding pattern. It has been suggested that posttranslational modifications, probably implicated in the stability or glycosylation of BclA are responsible for the multiple-band Western blotting patterns (Sylvestre *et al.* 2003). Our results with strains 5 to 11 emphasize the role of *bclA* gene-independent genetic factors in this phenomenon. In contrast to the mono- and disaccharide specific sera, the tetrasaccharide specific antibodies, which seem to bind to a complex epitope comprising all four sugar residues of the tetrasaccharide, showed preferential binding to the bands with low apparent molecular weight. This observation may indicate that the tetrasaccharide as a whole is better accessible in the low molecular weight BclA species. While anti-anthrose and anti-disaccharide antibodies are likely to bind, even if only the terminal residues are easily accessible, the anti-tetrasaccharide antibodies are likely to be much more sensitive to shielding of the deeper parts of the tetrasaccharide. Alternatively, glycosyl chains associated with high and low molecular weight species may slightly differ in their structure. Changes in the tri-rhamnose core structure, such as lack or substitution of one of the rhamnose residues are likely to affect the tetrasaccharide-specific antibodies but not those raised against anthrose or anthrose-rhamnose.

Even though the antibodies generated against anthrose-containing structures are not strictly specific for *B. anthracis*, they detect spore lysates of *B. anthracis* from a broad phylogenetic spectrum, thus representing a valuable immuno-capturing component for a highly sensitive spore detection system. Such sensitive immunoassays would be plagued by false-positive results but would afford a quick exclusion of negative samples from further analysis. Subsequent confirmatory expensive and time-consuming diagnostic tests with high specificity, such as real-time PCR or standard culture methods that focus on the vegetative form, are therefore minimized. Moreover it would be important to detect spores of *B. cereus*, since it is an opportunistic human pathogen and might be used as basis for engineered biological weapons.

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Tables and Figures

Table 1. Comparative analysis of the CLR composition of BclA from sequenced PCR products

Strains	Cluster [according to Pilo <i>et al.</i> 2008]	Origin	BclA protein of CLR		
			GPT repeats	BclA repeats [(GPT) ₅ GDTGTT]	GXX repeats [present once]
Nr. 1 JF3788	A4	Human Anthrax Switzerland from wool factory 1981	54	6	GAT GLT GPS GLG
Nr. 2 JF3853	A3B	Cattle central Switzerland 1952	57	5	GAT GLT GPS GLG GDT GTT
Nr. 3 JF3852	B2	Cattle Bern Switzerland 1953	21	1	GAT GLT GPS GLG
Nr. 4 JF3854	B2	Cattle central Switzerland 1957	21	1	GAT GLT GPS GLG
Nr. 5 JF3783	A4	Human Anthrax Switzerland from wool factory 1981	17	1	GAT GLT GPS GLG
Nr. 6 JF3784	A4	Air filter wool processing factory with outbreak 1981	17	1	GAT GLT GPS GLG
Nr. 7 JF3785	A4	goat hair, wool processing factory with outbreak 1981	17	1	GAT GLT GPS GLG
Nr. 8 JF3851	A4	unknown origin	17	1	GAT GLT GPS GLG
Nr. 9 JF3786	A4	goat hair, wool processing factory with outbreak 1981	17	1	GAT GLT GPS GLG
Nr. 10 JF3787	A4	goat hair, wool processing factory with outbreak 1981	17	1	GAT GLT GPS GLG
Nr. 11 A73	nd	unknown origin	17	1	GAT GLT GPS GLG

Fig. 1. Structure of the synthetic tetrasaccharide (**1**) of the major surface glycoprotein of *Bacillus anthracis* and deletion sequences of it: anthrose-rhamnose (**2**; disaccharide); anthrose (**3**; monosaccharide). Attachment via a pentenyl handle to KLH carrier protein afforded the conjugates for immunological studies.

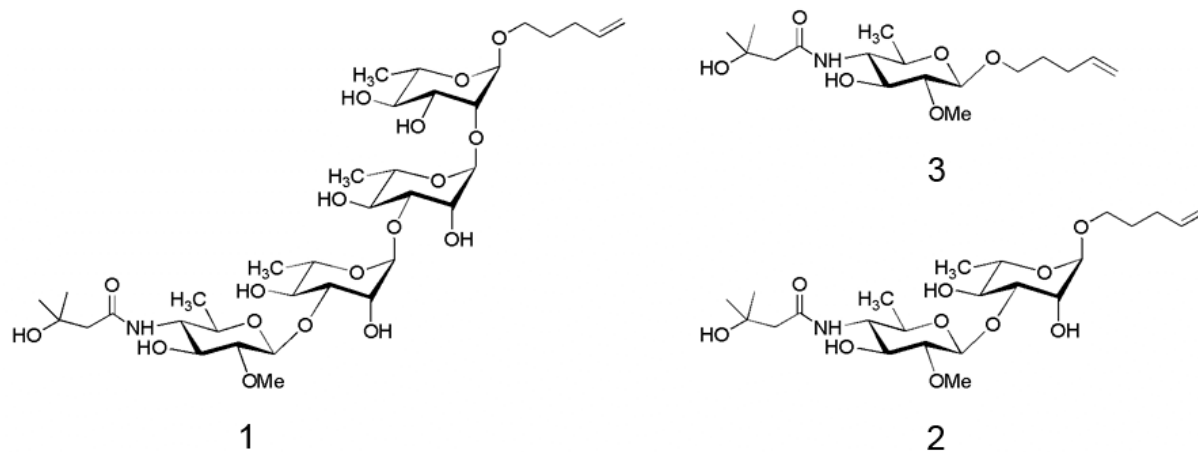


Fig. 2. Development of mouse IgG responses specific for the anthrose-containing tetrasaccharide, disaccharide and monosaccharide compounds shown in Fig. 1. Mice were immunized with monosaccharide-KLH (A) disaccharide-KLH (B) or tetrasaccharide-KLH (C) formulations. Shown are ELISA readouts obtained with serial dilution of mouse sera taken pre-immune (PI) and two weeks after the second, third or fourth immunization. Response patterns of individual tetrasaccharide specific mAbs (MTA1; MTA2; MTA3) are depicted in D. In (A) and (B) mean ELISA readouts \pm SD of sera from three mice are shown.

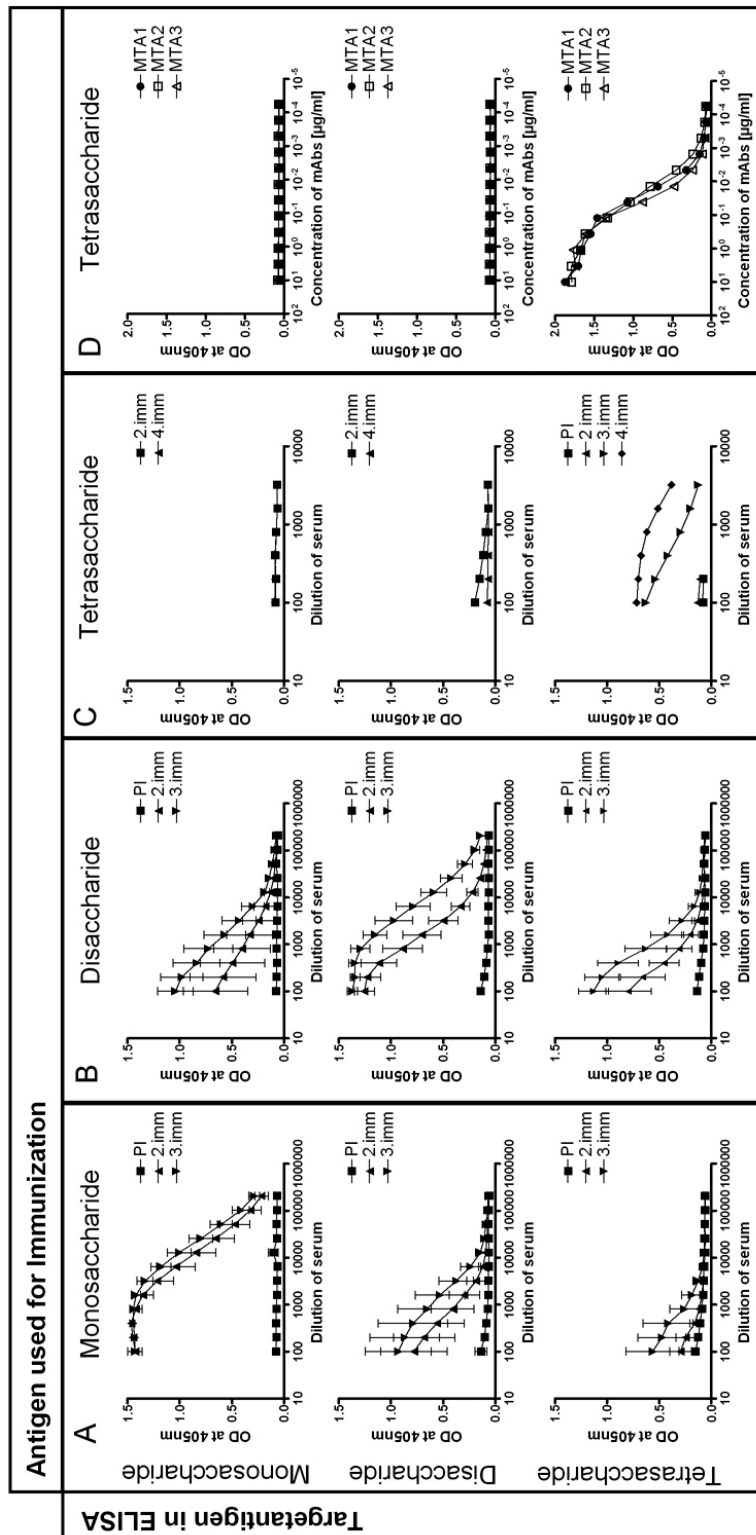


Fig. 3. Competition Western blot experiments with a set of overlapping synthetic sugars were used for epitope mapping. *B. anthracis* endospore suspensions were separated by SDS-10% PAGE under reducing conditions and blotted onto a nitrocellulose membrane. Anti-monosaccharide mouse sera (A), anti-disaccharide mouse sera (B) and mAb MTA1 (C) were pre-incubated with synthetic competitors and afterwards added to cut strips. After incubation with an alkaline phosphatase-conjugate blots were developed with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium to visualize bands. (D) Structures of different competitors used. Anthrose* = anthrose without the 3-hydroxy-3-methylbutamido chain. k = no competitor. a = 100 $\mu\text{g/ml}$ competitor. b = 1 $\mu\text{g/ml}$ competitor. Immune sera were used at a dilution of 1:2000 and mAb MTA1 at a concentration of 1 $\mu\text{g/ml}$.

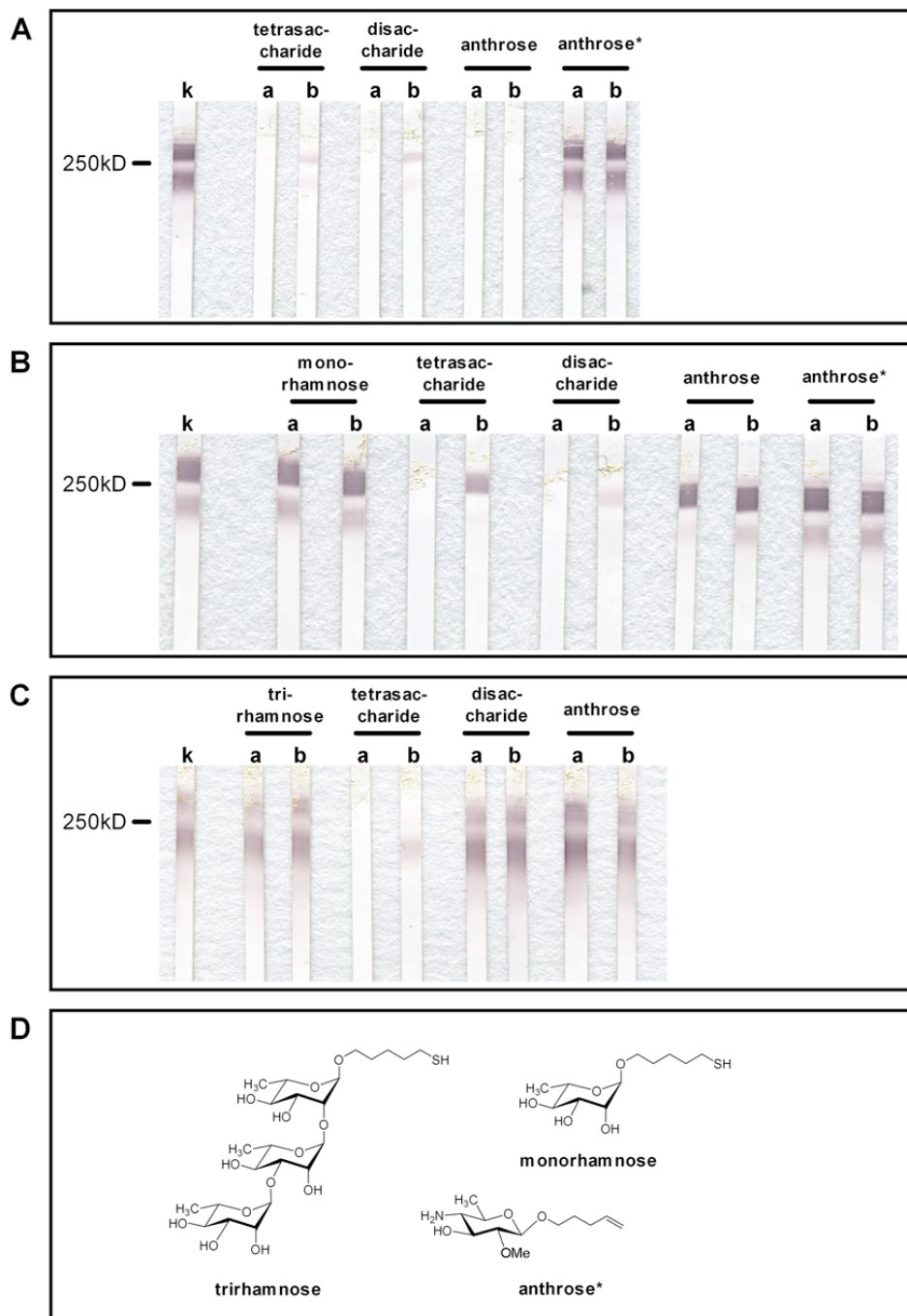


Fig. 4. (A) Differences in the size of the *bclA* gene in 11 *B. anthracis* strains (lanes 1 to 11; see also table 1). The sequences encoding the collagen-like regions were amplified by PCR and analyzed by 2% agarose gel electrophoresis. Molecular size markers are indicated in base pairs (bp). (B - E) Western blot analysis with spore lysates of the 11 *B. anthracis* strains. Endospore lysates were separated by SDS-10% PAGE and blotted onto a nitrocellulose membrane. Blots were incubated with anti-tetrasaccharide mAb MTA1 [0.01 μ g/ml] (B), with disaccharide- (C), anthrose- (D) and *B. thuringiensis*-antisera (E). All immune sera were used at a dilution of 1:1000 and blots were developed using the ECL system. The sizes of the molecular weight markers are given in kDa. (F) EZBlue™ (Sigma) protein staining of the 11 *B. anthracis* separated endospore lysates on a SDS-10% gel.

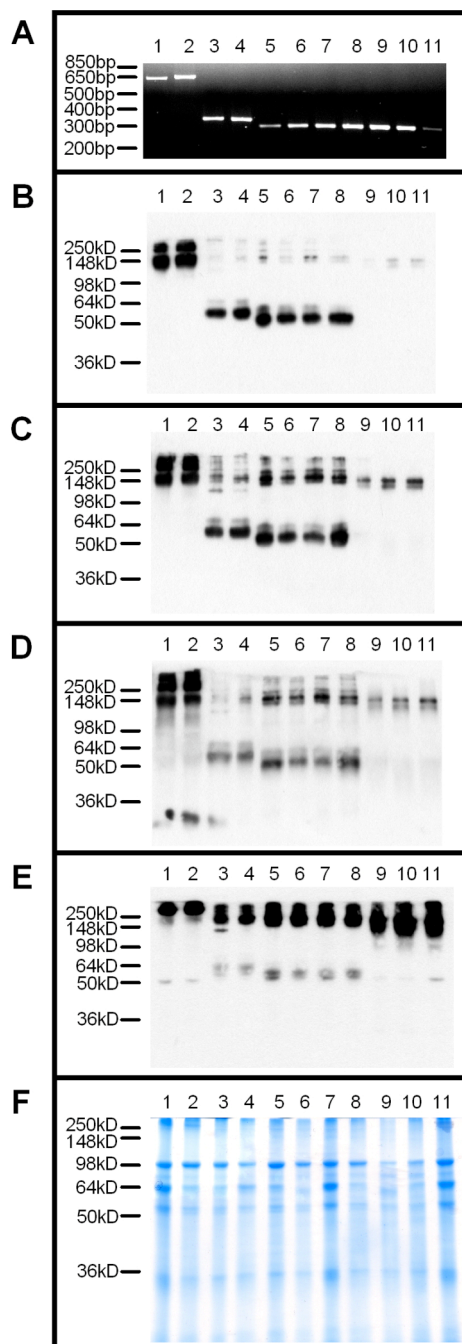
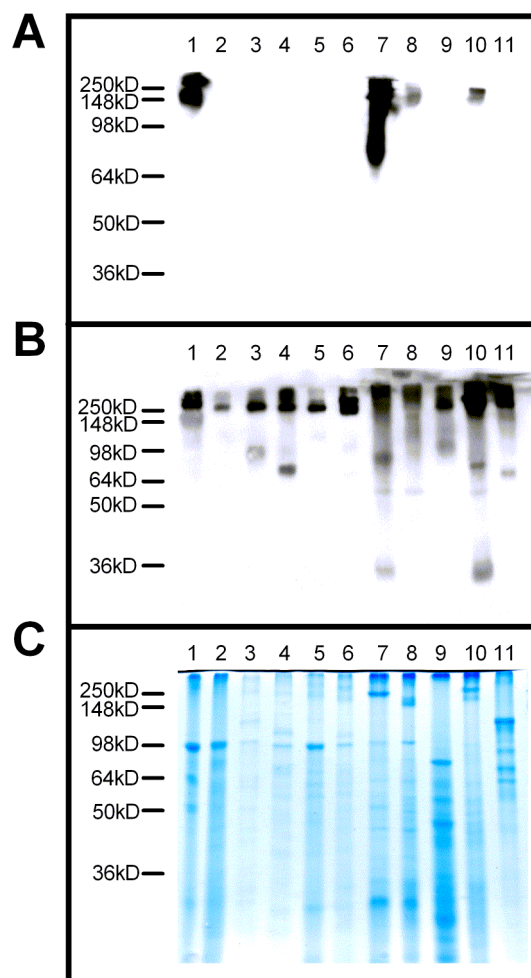


Fig. 5. Western blot analysis of the reactivity of anti-tetrasaccharide mAb MTA1 (A) and anti-*B. thuringiensis* antiserum (B) with spore lysates. Total spore-lysates of *B. anthracis* strain 1 (lane 1), *B. cereus* F4370/75 (lane 2), *B. cereus* m1545 (lane 3), *B. cereus* m1564 (lane 4), *B. cereus* m1293 (lane 5), *B. cereus* F4810/72 (lane 6), *B. cereus* ATCC 10876 (lane 7), *B. cereus* ATCC 13061 (lane 8), *B. cereus* ATCC 14579 (lane 9), *B. cereus* ATCC 33019 (lane 10) and *B. thuringiensis* ATCC 29730 (lane 11) were separated by SDS-10% PAGE and blotted onto a nitrocellulose membrane. Anti-*B. thuringiensis* antiserum was used at a dilution of 1:1000 and the mAb at a concentration of 0.01 μ g/ml. (C) EZBlueTM (Sigma) protein staining of the 11 *Bacillus* species separated on a SDS-10% gel. The sizes of the molecular mass markers are given in kDa.



Supplementary material

General Methods: All chemicals used were reagent grade and used as supplied except where noted. Dichloromethane (CH_2Cl_2) was purchased from JT Baker and purified by a Cycle-Tainer Solvent Delivery System. Pyridine and acetonitrile were refluxed over calcium hydride and distilled prior to use. Analytical thin-layer chromatography was performed on E. Merck silica gel 60 F254 plates (0.25 mm). Compounds were visualized by dipping the plates in a cerium sulfate ammonium molybdate solution or a sulphuric acid/methanol solution (for fully deprotected compounds) followed by heating. Liquid chromatography was performed using forced flow of the indicated solvent on Sigma H-type silica (10–40 mm). HPLC purifications were performed by a Waters system 2420, using a reversed-phase C18 column. ^1H NMR spectra were obtained on a Varian VXR-300 (300 MHz), Bruker-600 (600 MHz), and are reported in parts per million (δ) relative to CHCl_3 ($\delta = 7.26$ ppm) or in the case of CD_3OD as solvent relative to TMS ($\delta = 0.00$ ppm). Coupling constants (J) are reported in Hertz (Hz). ^{13}C NMR spectra were obtained on a Varian VXR-300 (75 MHz), Bruker-600 (150 MHz) and are reported in δ relative to CDCl_3 ($\delta = 77.0$ ppm) as an internal reference or to TMS ($\delta = 0.00$ ppm). For α/β mixtures we abstained from measuring $[\alpha]_D$ values.

Pent-4-enyl 4-Azido-3-*O*-benzyl-4,6-dideoxy-2-*O*-levulinoyl- β -D-glucopyranoside (2): 4-Azido-3-*O*-benzyl-4,6-dideoxy-2-*O*-levulinoyl- β -D-glucopyranosyl Trichloroacetimidate¹ **1** (310 mg, 0.594 mmol, 1.0 equiv.) was azeotroped three times with toluene and dried in vacuo for 30 min. CH_2Cl_2 (7 mL) was added as well as 4-pentenol (205 mg, 2.380 mmol, 4.0 equiv.). The solution was cooled to -35 °C. TMSOTf (13 mg, 11 μL , 0.059 mmol, 0.1 equiv.) was added and the reaction mixture stirred for 40 min. Then, the reaction was quenched by addition of pyridine (0.1 mL). Column chromatography on silica gel (cyclohexane/EtOAc, 3:1) yielded 225 mg (85%) as a colorless solid: $[\alpha]_D = 109.1$ ($c = 1.0$, CHCl_3). IR (thin film, CHCl_3): 3005, 2933, 2872, 2103, 1744, 1713, 1415, 1359, 1154, 1072 cm^{-1} . ^1H NMR (CDCl_3 , 300 MHz): $\delta = 1.35$ (d, $J = 5.7$ Hz, 3H), 1.61–1.73 (m, 2H), 2.03–2.18 (m, 2H), 2.15 (s, 3H), 2.48–2.53 (m, 2H), 2.67–2.72 (m, 2H), 3.16–3.26 (m, 2H), 3.39–3.56 (m, 2H), 3.78–3.85 (m, 1H), 4.29 (d, $J = 7.8$ Hz, 1H), 4.69 (d, $J = 11.1$ Hz, 1H), 4.78 (d, $J = 11.4$ Hz, 1H), 4.94–5.03 (m, 3H), 5.74–5.83 (m, 1H), 7.26–7.35 (m, 5H) ppm. ^{13}C NMR (CDCl_3 , 75 MHz): $\delta = 18.6, 28.1, 28.9, 30.1, 30.1, 38.0, 67.9, 69.3, 70.9, 73.9, 75.0, 81.4, 101.0, 115.1, 128.1, 128.4, 128.6, 137.8, 138.2, 171.5, 206.3$ ppm. MALDI-HRMS: m/z $[\text{M} + \text{Na}]^+$ calc.468.2105, obsd. 468.2097.

¹ D.B. Werz, P.H. Seeberger, *Angew. Chem. Int. Ed.* **2005**, *44*, 6315-6318.

Pent-4-enyl 4-Azido-3-O-benzyl-4,6-dideoxy- β -D-glucopyranoside (3): The glycoside **2** (225 mg, 0.505 mmol, 1.0 equiv.) was dissolved in CH₂Cl₂ (5 mL), pyridine (133 μ L), AcOH (66 μ L), and hydrazine monohydrate (3.5 μ L, 1.010 mmol, 2.0 equiv.) were added. The reaction mixture was stirred for 1 h at room temperature. The reaction was quenched by addition of acetone (1 mL) and concentrated. Column chromatography on silica gel (cyclohexane/EtOAc 5:1) yielded 121 mg (69 %) of a colorless oil; $[\alpha]_D = 39.6$ ($c = 0.5$, CHCl₃). IR (thin film, CHCl₃): 3008, 2936, 2110, 1454, 1354, 1065 cm⁻¹. ¹H NMR (CDCl₃, 300 MHz): $\delta = 1.35$ (d, $J = 6.0$ Hz, 3H), 1.67–1.79 (m, 2H), 2.11–2.18 (m, 2H), 2.48 (s, 1H), 3.11–3.3.72 (m, 2H), 3.41–3.58 (m, 3H), 3.87–3.94 (m, 1H), 4.19 (d, $J = 7.5$ Hz, 1H), 4.84 (d, $J = 11.1$ Hz, 1H), 4.95–5.07 (m, 3H), 5.77–5.87 (m, 1H), 7.30–7.44 (m, 5H) ppm. ¹³C NMR (CDCl₃, 75 MHz): $\delta = 18.6, 29.0, 30.2, 60.6, 67.4, 69.3, 70.3, 75.4, 82.7, 84.2, 103.2, 114.9, 127.8, 128.2, 128.3, 137.9$ ppm. MALDI-HRMS: m/z [M + Na]⁺ calc. 347.1840, obsd. 347.1834.

Pent-4-enyl 4-Azido-3-O-benzyl-4,6-dideoxy-2-O-methyl- β -D-glucopyranoside (4): The glycoside **3** (120 mg, 0.345 mmol, 1.0 equiv.) was dissolved in DMF (6 mL) and cooled to 0 °C. Sodium hydride (23 mg, 0.575 mmol, 1.6 equiv.) was added, then methyl iodide (125 mg, 39 μ L, 0.881 mmol, 2.6 equiv.) was added and the mixture was stirred over night. The reaction mixture was poured into water and extracted twice with EtOAc. The combined organic phases were washed with brine, dried over MgSO₄ and concentrated. Column chromatography on silica gel (cyclohexane/EtOAc 10:1) was performed to yield 119 mg (95 %) as a colorless oil: $[\alpha]_D = 37.4$ ($c = 0.5$, CHCl₃). IR (thin film, CHCl₃): 2928, 2110, 1454, 1354, 1261, 1088, 1029 cm⁻¹. ¹H NMR (CDCl₃, 300 MHz): $\delta = 1.33$ (d, $J = 5.7$ Hz, 3H), 1.71–1.77 (m, 2H), 2.14–2.21 (m, 2H), 3.09–3.22 (m, 3H), 3.40 (t, $J = 9.1$ Hz, 1H), 3.47–3.55 (m, 1H), 3.62 (s, 3H), 3.88–3.93 (m, 1H), 4.23 (d, $J = 8.1$ Hz, 1H), 4.80 (d, $J = 10.5$ Hz, 1H), 4.91 (d, $J = 10.5$ Hz, 1H), 4.98–5.08 (m, 2H), 5.76–5.87 (m, 1H), 7.31–7.44 (m, 5H) ppm. ¹³C NMR (CDCl₃, 75 MHz): $\delta = 18.6, 29.0, 30.2, 60.6, 60.6, 67.5, 69.3, 70.3, 75.4, 82.7, 84.2, 103.2, 114.9, 127.8, 128.2, 128.3, 137.9$ ppm. MALDI-HRMS: m/z [M + Na]⁺ calc. 384.1894, obsd. 384.1895.

Pent-4-enyl 4-Amino-4,6-dideoxy-2-O-methyl- β -D-glucopyranoside (5): The glucoside **4** (118 mg, 0.326 mmol, 1.0 equiv.) was dissolved under an Ar atmosphere in THF (7 mL) and ammonia was condensed into the reaction vessel at -78 °C until the mixture reached a volume of about 20 mL. Small pieces of carefully washed sodium were added to the reaction vessel affording a solution with a deep blue color. After 30 min the reaction was quenched by adding some drops of methanol. Again, small pieces of sodium were added until the blue color persists for at least 30 min. The reaction was quenched by adding several mL of methanol. The dry ice bath was removed, the

reaction mixture was allowed to warm to room temperature in order to evaporate the ammonia. Amberlite IR-120 acidic resin (washed) was added until pH 7 was reached. The mixture was filtered, rinsed and concentrated. The crude product was purified by column chromatography using a reverse phase column C 18 silica gel (MeOH/H₂O). The product containing fractions were combined and concentrated affording 36 mg (44%) of a colorless solid; $[\alpha]_D = -30.4$ ($c = 0.5$, CHCl₃). IR (thin film, CHCl₃): 3596, 3008, 1602, 1373, 1173, 1078, 910 cm⁻¹. ¹H NMR (CDCl₃, 300 MHz): $\delta = 1.27$ (d, $J = 6.0$ Hz, 3H), 1.67–1.77 (m, 3H), 2.10–2.18 (m, 2H), 2.48 (t, $J = 9.6$ Hz, 1H), 2.96 (d, $J = 7.8$ Hz, 1H), 3.15–3.26 (m, 2H), 3.42–3.58 (m, 2H), 3.60 (s, 3H), 3.87–3.94 (m, 1H), 4.25 (d, $J = 8.1$ Hz, 1H), 4.94–5.05 (m, 2H), 5.76–5.86 (m, 1H) ppm. ¹³C NMR (CDCl₃, 75 MHz): $\delta = 17.7, 28.8, 30.1, 58.2, 60.5, 69.0, 72.9, 75.7, 83.6, 103.2, 114.8, 138.0$ ppm. MALDI-HRMS: m/z [M + H]⁺ calc. 246.1700, obsd. 246.1698.

***n*-Pentenyl 4,6-Dideoxy-4-(3-hydroxy-3-methylbutanamido)-2-*O*-methyl- β -D-glucopyranoside (6):** The glucoside **5** (30 mg, 0.122 mmol) was dissolved in DMF (4 mL) and 3-hydroxy-3-methylbutyric acid (29 mg, 0.245 mmol) in 0.5 mL of DMF were added. HATU (88 mg, 0.232 mmol), followed by *N,N*-diisopropylethylamine (63 mg, 81.0 μ L, 0.489 mmol) was added yielding a slightly yellow solution. The mixture is stirred at room temperature for 2 h. All volatiles were removed *in vacuo*. The residue was purified by column chromatography using silica gel (dichloromethane/methanol 4:1) and further purified by using a reverse phase column C18 silica gel (MeOH/H₂O). The product containing fractions were combined and concentrated affording 32 mg (76%) of a colorless solid; $[\alpha]_D = -26.8$ ($c = 0.5$, CHCl₃). IR (thin film, CHCl₃): 3430, 3008, 2934, 1731, 1664, 1251, 1374, 1076 cm⁻¹. ¹H NMR (CDCl₃, 300 MHz): $\delta = 1.25$ –1.31 (m, 9H), 1.70–1.78 (m, 2H), 2.11–2.19 (m, 2H), 2.38 (s, 2H), 3.00 (t, $J = 8.1$ Hz, 1H), 3.27 (s, 1H), 3.43–3.53 (m, 3H), 3.61 (s, 3H), 3.65 (d, $J = 9.0$ Hz, 1H), 3.73 (s, 1H), 3.87–3.95 (m, 1H), 4.25 (d, $J = 7.8$ Hz, 1H), 4.95–5.06 (m, 2H), 5.75–5.86 (m, 1H), 6.15 (d, $J = 8.7$ Hz, 1H) ppm. ¹³C NMR (CDCl₃, 75 MHz): $\delta = 18.1, 29.0, 29.3, 29.8, 30.3, 48.7, 57.0, 60.7, 69.1, 69.9, 70.7, 74.0, 83.9, 103.0, 114.9, 137.9, 172.8$ ppm. MALDI-HRMS: m/z [M + Na]⁺ calc. 368.2049, obsd. 368.2038.

Pent-4-enyl 4-Azido-3-*O*-benzyl-6-desoxy-2-*O*-methyl- β -D-glucopyranosyl-(1 \rightarrow 3)-2-*O*-benzoyl-4-*O*-benzyl- α -L-rhamnopyranoside (8): 4-Azido-3-*O*-benzyl-6-desoxy-2-*O*-methyl- β -D-glucopyranosyl-(1 \rightarrow 3)-2-*O*-benzoyl-4-*O*-benzyl- α -L-rhamnopyranosyl Trichloroacetimidate **7** (85 mg, 0.109 mmol, 1.0 equiv.) was azeotroped three times with toluene and dried *in vacuo* for 30 min. CH₂Cl₂ (2.5 mL) was added as well as 4-pentenol (38 mg, 0.441 mmol, 4.0 equiv.). The solution was cooled to -30 °C. TMSOTf (2.5 mg, 2.0 μ L, 0.011 mmol, 0.1 equiv.) was added and the reaction mixture stirred for 30 min. Then, the reaction was quenched by addition of pyridine

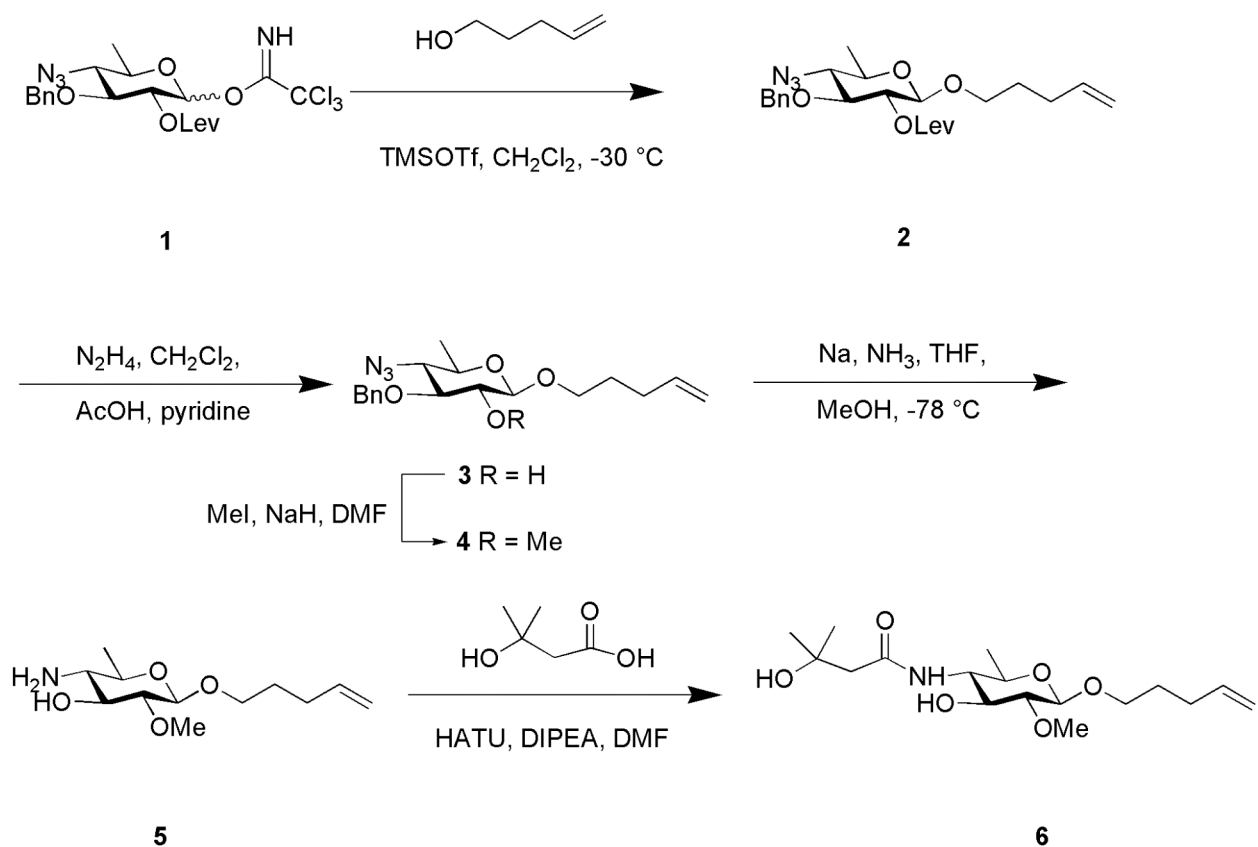
(0.1 mL). Column chromatography on silica gel (cyclohexane/EtOAc, 10:1) yielded 48 mg (63%) as a colorless solid: $[\alpha]_D = 48.4$ ($c = 0.5$, CHCl_3). IR (thin film, CHCl_3): 2936, 2110, 1720, 1270, 1069 cm^{-1} . ^1H NMR (CDCl_3 , 300 MHz): $\delta = 1.23$ (d, $J = 5.9$ Hz, 3H), 1.38 (d, $J = 6.2$ Hz, 3H), 1.65–1.75 (m, 2H), 2.10–2.18 (m, 2H), 3.00–3.20 (m, 3H), 3.28–3.44 (m, 2H), 3.52 (s, 3H), 3.58–3.71 (m, 2H), 3.80–3.86 (m, 1H), 4.28 (dd, $J = 3.5, 9.4$ Hz, 1H), 4.60 (d, $J = 7.8$ Hz, 1H), 4.70 (d, $J = 10.4$ Hz, 1H), 4.78 (d, $J = 10.8$ Hz, 1H), 4.82 (d, $J = 12.5$ Hz, 1H), 4.86 (d, $J = 13.0$ Hz, 1H), 4.96–5.08 (m, 3H), 5.36 (dd, $J = 1.8, 3.4$ Hz, 1H), 5.75–5.89 (m, 1H), 7.26–7.49 (m, 12H), 7.56–7.61 (m, 1H), 8.07 (d, $J = 7.2$ Hz, 2H) ppm. ^{13}C NMR (CDCl_3 , 75 MHz): $\delta = 18.1, 18.4, 28.7, 30.3, 60.5, 67.2, 67.4, 67.6, 70.4, 73.3, 75.0, 75.3, 76.4, 80.9, 82.7, 84.4, 97.2, 102.9, 114.9, 127.7, 127.8, 128.1, 128.1, 128.2, 128.3, 128.4, 129.6, 130.2, 132.9, 137.8, 137.9, 138.0, 165.7$ ppm. MALDI-HRMS: m/z $[\text{M} + \text{Na}]^+$ calc. 724.3205, obsd. 724.3218.

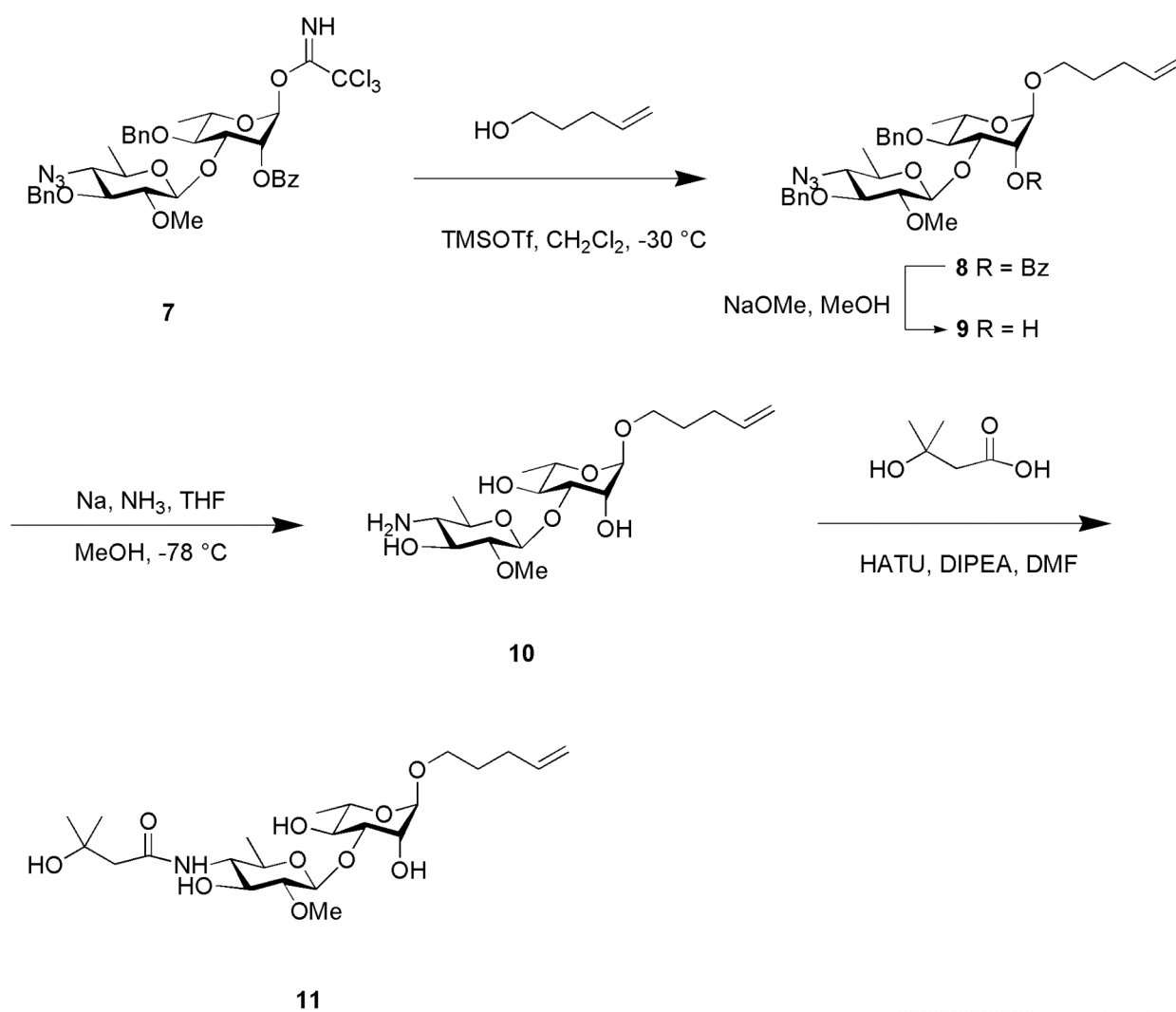
Pent-4-enyl 4-Azido-3-O-benzyl-6-desoxy-2-O-methyl- β -D-glucopyranosyl-(1 \rightarrow 3)-O-benzyl- α -L-rhamnopyranoside (9): Disaccharide **8** 48 mg, (68.4 μmol) was dissolved in MeOH (8 mL) and NaOMe (0.6 mL, 0.5 M in MeOH) was added. The mixture was stirred over night. The solution was neutralized by treating with Amberlite IR-120, filtered and washed. The solvent was removed under reduced pressure, the residue was purified by flash chromatography (cyclohexane/EtOAc 4:1) to afford: 41 mg (68 μmol , quant.) as a colorless oil: $[\alpha]_D = 5.2$ ($c = 0.5$, CHCl_3). IR (thin film, CHCl_3): 3008, 2911, 2111, 1453, 1384, 1093, 1064 cm^{-1} . ^1H NMR (CDCl_3 , 300 MHz): $\delta = 1.30$ –1.34 (m, 6H), 1.62–1.72 (m, 2H), 2.05–2.15 (m, 2H), 2.81 (s, 1H), 3.09–3.26 (m, 3H), 3.37–3.44 (m, 2H), 3.48–3.52 (m, 1H), 3.57 (s, 3H), 3.64–3.78 (m, 2H), 3.99–4.01 (m, 2H), 4.54 (d, $J = 7.8$ Hz, 1H), 4.61 (d, $J = 10.8$ Hz, 1H), 4.78–4.93 (m, 4H), 4.96–5.05 (m, 2H), 5.74–5.85 (m, 1H), 7.26–7.43 (m, 10H) ppm. ^{13}C NMR (CDCl_3 , 75 MHz): $\delta = 18.1, 18.7, 28.7, 30.4, 60.8, 66.9, 67.2, 67.3, 70.5, 70.6, 74.9, 75.5, 79.8, 81.2, 82.9, 84.2, 99.0, 102.9, 114.9, 127.7, 127.8, 128.0, 128.3, 128.4, 137.7, 137.9, 138.2$ ppm. MALDI-HRMS: m/z $[\text{M} + \text{Na}]^+$ calc. 620.2942, obsd. 620.2942.

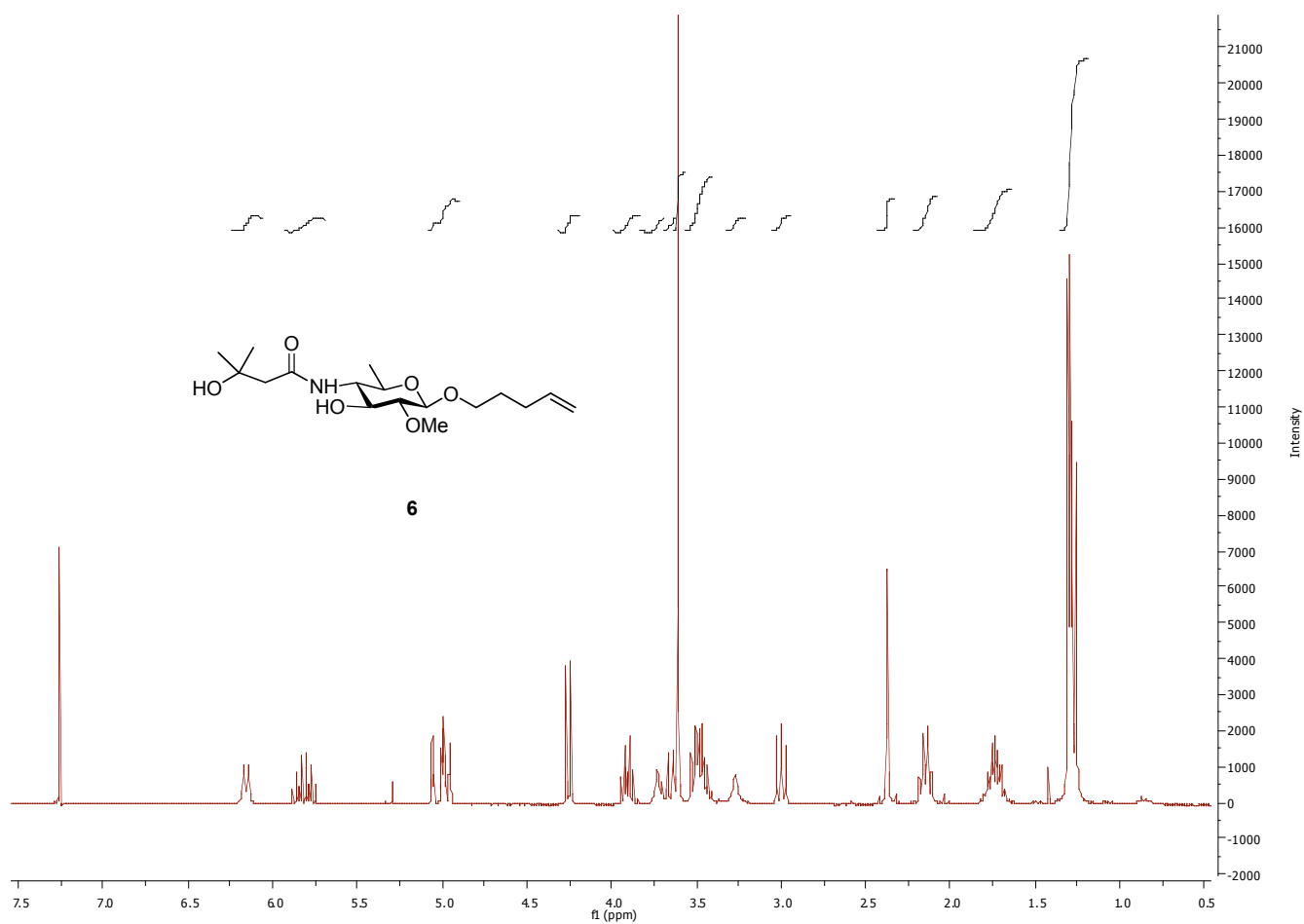
Pent-4-enyl 4-Amino-4,6-dideoxy-2-O-methyl- β -D-glucopyranosyl-(1 \rightarrow 3)- α -L-rhamnopyranoside (10): The glucoside **9** (40 mg, 66.9 μmol) was dissolved under an Ar atmosphere in THF (7 mL) and ammonia was condensed into the reaction vessel at -78 $^\circ\text{C}$ until the mixture reached a volume of about 20 mL. Small pieces of carefully washed sodium were added to the reaction vessel affording a solution with a deep blue color. After 30 min the reaction was quenched by adding some drops of methanol. Again, small pieces of sodium were added until the blue color persists for at least 30 min. The reaction was quenched by adding several mL of

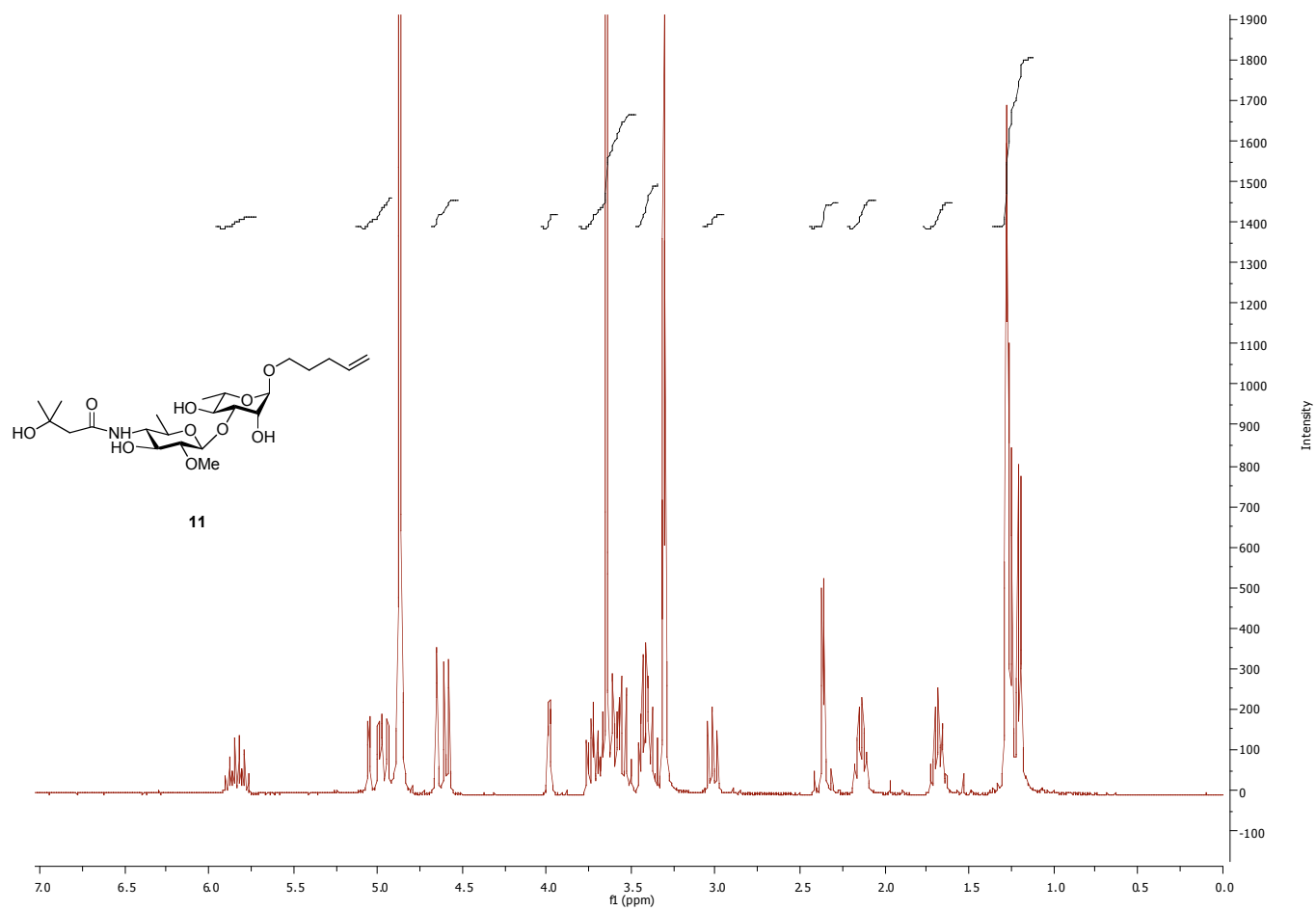
methanol. The dry ice bath was removed, the reaction mixture was allowed to warm to room temperature in order to evaporate the ammonia. Amberlite acidic resin (washed) was added until pH 7 was reached. The mixture was filtered, rinsed and concentrated. The crude product was purified by column chromatography using a reverse phase column C 18 silica gel (MeOH/H₂O). The product containing fractions were combined and concentrated affording 21 mg (80%) of a colorless solid; $[\alpha]_D = -51.6$ ($c = 0.5$, CHCl₃). IR (thin film, CHCl₃): 3590, 3008, 2927, 1449, 1382, 1056 cm⁻¹. ¹H NMR (CD₃OD, 300 MHz): $\delta = 1.25$ (d, $J = 6.3$ Hz, 6H), 1.66–1.73 (m, 3H), 2.11–2.18 (m, 2H), 2.36 (t, $J = 9.6$ Hz, 1H), 2.96 (t, $J = 8.4$ Hz, 1H), 3.16–3.33 (m, 2H), 3.35–3.44 (m, 1H), 3.47–3.76 (m, 7H), 3.98–3.99 (m, 1H), 4.58 (d, $J = 7.8$ Hz, 1H), 4.65 (s, 1H), 4.94–5.06 (m, 2H), 5.78–5.88 (m, 1H) ppm. ¹³C NMR (CD₃OD, 75 MHz): $\delta = 17.8, 18.2, 29.6, 31.2, 59.5, 60.7, 67.6, 69.6, 71.7, 72.7, 73.2, 76.7, 81.4, 85.0, 101.0, 105.4, 114.9, 138.9$ ppm. MALDI-HRMS: m/z [M + H]⁺ calc. 414.2098, obsd. 414.2097.

***n*-Pentenyl 14,6-Dideoxy-4-(3-hydroxy-3-methylbutanamido)-2-*O*-methyl- β -D-glucopyranosyl-(1 \rightarrow 3)- α -L-rhamnopyranoside (11):** The glucoside **10** (20 mg, 51.1 μ mol) was dissolved in DMF (4 mL) and 3-hydroxy-3-methylbutyric acid (12 mg, 102 μ mol) in 0.5 mL of DMF were added. HATU (37 mg, 97 μ mol), followed by *N,N*-diisopropylethylamine (26 mg, 33 μ L, 201 mmol) was added yielding a slightly yellow solution. The mixture is stirred at room temperature for 2 h. All volatiles were removed *in vacuo*. The residue was purified by column chromatography using silica gel (dichloromethane/methanol 4:1) and further purified by using a reverse phase column C 18 silica gel (MeOH/H₂O). The product containing fractions were combined and concentrated affording 21 mg (84%) of a colorless solid: $[\alpha]_D = -50.0$ ($c = 0.5$, CHCl₃). IR (thin film, CHCl₃): 3429, 3008, 2928, 1663, 1520, 1383, 1075 cm⁻¹. ¹H NMR (CD₃OD, 300 MHz): $\delta = 1.20$ (d, $J = 6.0$ Hz, 3H), 1.25–1.28 (m, 9H), 1.66–1.73 (m, 2H), 2.11–2.18 (m, 2H), 2.36 (d, $J = 3.0$ Hz, 2H), 3.02 (t, $J = 8.4$ Hz, 1H), 3.34–3.45 (m, 2H), 3.50–3.76 (m, 8H), 3.97–3.99 (m, 1H), 4.59 (d, $J = 8.1$ Hz, 1H), 4.87 (s, 1H), 4.95–5.05 (m, 2H), 5.79–5.88 (m, 1H) ppm. ¹³C NMR (CD₃OD, 75 MHz): $\delta = 17.7, 18.2, 29.3, 29.4, 29.6, 31.2, 57.7, 60.8, 67.5, 69.6, 70.3, 71.7, 72.6, 74.6, 81.3, 85.1, 101.0, 105.2, 114.9, 138.8, 174.0$ ppm. MALDI-HRMS: m/z [M + Na]⁺ calc. 514.2623, obsd. 514.2629.

Scheme 1. Synthesis of anthrose **6**

Scheme 2. Synthesis of disaccharide **11**

¹H NMR (CDCl₃, 300 MHz) spectrum of compound **6**

^1H NMR (CD_3OD , 300 MHz) spectrum of compound **11**

CHAPTER 8: GENERAL DISCUSSION AND CONCLUSIONS

8.1 General Discussion

8.1.1 Malaria Control, a Changing Concept from Prevalence-based to Disease-based Vaccines

In ancient times malaria was understood as God's punishment for human sins. The idea that the environment could affect human health by means of *miasms* associated with swamps was also applied to malaria. In the age of Renaissance the concept of *contagion* was introduced, which was later on replaced by that of *infectious agents*. In the 20th century a better understanding of the pathological aspects of the disease allowed to consider severe malaria as an *immune endotheliopathy*. At present a novel concept about malaria is assumed: malaria as the result of disequilibrium of the system 'human-plasmodium-mosquito' (1). If this system is maintained in equilibrium, malaria does not ensue, and the result is asymptomatic plasmodium infection. Epidemiological studies show that asymptomatic malaria infections outnumber the symptomatic cases. These observations lead to the conclusion that the state of equilibrium or adaptation is the rule in human host-plasmodium relationships. Clinical manifestations, particularly the rare occurrence of severe and complicated forms of malaria, can be considered as a stage before equilibrium is reached or is lost either due to an overgrowth of the parasite and/or to dysfunction of the immune system (1).

The currently available strategies for malaria control rely on destruction by fighting malaria parasites with drugs and the anopheline vectors with insecticides (2, 3). This combined approach proved remarkably effective in Europe and North America, whereas malaria continues to represent a huge burden in sub-Saharan Africa, Asia and South and Central America mainly due to drug-resistant parasites and insecticide resistant vectors (4). Pyrethroid-treated bednets are the mainstay of malaria control programs. Pyrethroids are highly effective insecticides showing low mammalian toxicity levels and currently the only class of insecticide available for use on bednets (5). Therefore the emergence of pyrethroid resistance threatens the successful use of insecticide-treated materials (6, 7). Resistance of plasmodium to antimalarial therapy has been described in endemic areas as a consequence of drug pressure (8). There is no doubt that the speed drug resistance occurs is far faster than that new antimalarial drugs are developed. It is possibly considered unreasonable to base malaria control on the destruction of plasmodia and mosquitoes, species extremely much older and, therefore, more selected and adaptable, than human beings (1).

An effective malaria vaccine is not yet available, in part because of the high antigenic and biological complexity of the parasite; the lack of adequate models for the infection; the insufficient understanding of anti-plasmodial immunity; the unavailability of reliable parameters and tests for assessing protective immunity; the difficulty of identifying, selecting and producing the appropriate protective antigens from a parasite with a genome of well over five thousand genes (9); the frequent need to enhance the immunogenicity of purified antigens through the use of novel adjuvants or delivery systems; and the genetic restriction of immune responses of the target-population (1, 10).

The traditional approach to develop malaria vaccines has focused on targeting one of the different stages of parasite development, whether the pre-erythrocytic, the intra-erythrocytic or the sexual stage. Pre-erythrocytic vaccine strategies aim to generate antibodies able to neutralize sporozoites and prevent them from invading hepatocytes, as well as to elicit a cell-mediated immune response able to interfere with the intra-hepatic schizogony. Asexual blood-stage vaccine strategies aim to elicit antibodies that will inactivate merozoites and target malarial antigens expressed on the RBC surface through antibody-dependent cellular cytotoxicity and complement lysis; and also are meant to elicit T cell responses able to inhibit the development of the parasite in RBCs (10). Transmission-blocking vaccines are aimed to induce antibodies against the sexual stage antigens in order to prevent the development of infectious sporozoites in the salivary glands of the mosquitoes (11). They are intended to protect communities from infection, not individuals from disease.

The usually expected outcome of malaria vaccines has been the reduction of local malaria prevalence. Therefore, first attempts in malaria vaccine development aimed at sterile protection from infection, which can only be achieved with a pre-erythrocytic vaccine. Indeed, irradiated sporozoites (12) and the RTS,S/AS02A (13) vaccine, which consists of a *P. falciparum* CSP polypeptide fused to the recombinant S antigen of hepatitis B virus, succeeded in conferring sterile protection to immunized volunteers experimentally infected after vaccination. However, the feasibility of a sporozoite vaccine remains doubtful and RTS,S/AS02A failed to show sterile protection in the field (14). One objection raised against sporozoite, liver-stage and sexual stage vaccines is that by interrupting transmission and preventing blood-stage infection, they may paradoxically prevent the acquisition of immunity in naive children or allow the waning of acquired immunity in adults, leading to more severe malaria once breakthrough infections establish (15).

Therefore the expected outcome of modern malaria vaccine development has shifted from protecting against the parasite to surviving with the parasite but without experiencing the noxious effects it can cause. The aim of such an anti-disease vaccine would be to prevent both parasite overgrowth and the over-stimulation of the immune system, the two causes of mortality in malaria. Blood-stage antigens and parasite toxins may represent the key components of an effective anti-

disease vaccine that permits the establishment of the natural equilibrium in the ‘human-plasmodium-mosquito’ system.

Asexual blood-stage vaccines are aimed to primarily protect against severe malaria disease, and not against infection, on the assumption that inhibition of parasite invasion cycles will lead to reduced parasite burden and decreased morbidity and mortality (16). Nonetheless, an alternative view is that reducing the replication of bloodstage parasites will not necessarily reduce morbidity or mortality, because host immune responses, which can be non-linear with respect to parasite densities, are important determinants of these events (17). Merozoite surface proteins are a major focus of research for blood-stage vaccines and the favoured candidates for future trials are MSP1 and AMA1. Other merozoite proteins (MSP3, MSP4, MSP5, RAP2, D13 and GLURP) are at an earlier stage along the vaccine development path (1, 18).

As noted, the primary objective of a malaria vaccine is to reduce morbidity and mortality. Therefore, the novel concept of developing an anti-toxic vaccine is justified on the extensive evidence that malaria morbidity and fatalities have a toxic basis. However, it has been argued that anti-toxic vaccines might aggravate disease by inhibiting the acute-phase responses that limit parasite replication (19). Putative malaria toxins include GPI (sect. 1.2.2), haemozoin (an insoluble digestion product of trophozoites comprising detoxified haemoglobin, as well as remnants of host and parasite membranes) and anti-malaria IgE-antigen complexes (20-22). Anti-GPI antibodies have been proposed as mediators of anti-disease immunity and it is controversially debated, whether GPI could be a suitable component of a malaria vaccine (23).

8.1.2 A Dualistic Approach to study the Relevance of Anti-GPI Immune Response for Protection against Malaria

The pathological significance of GPIs has to be confirmed by studies of pathophysiology and natural immunity using more accurate analysis. The carbohydrate microarray based on synthetic *Pf*GPI glycans developed in this thesis represented a dualistic device to test the hypothesis that this molecule is causally involved in malaria pathogenesis: natural immunity was analysed with the array by assessing levels and fine specificities of anti-GPI antibodies in healthy and malaria exposed individuals. In addition the microarray served as immunoassay for the selection of GPI specific B cells. The generated monoclonal anti-GPI antibodies also emerged as a dualistic tool to experimentally investigate the potential of anti-GPI antibodies as vaccine component and to study the GPI-anchored proteome of *Apicomplexa* species.

Understanding the relationship between GPI structures and anti-GPI antibody-binding activities is needed for a rational design of GPI-based vaccine candidates. The high throughput

serum screening strategy revealed a structure-dependent binding of anti-GPI antibodies to the GPI fragments. Anti-Man₃ GPI response was associated with malaria-exposure whereas anti-Man₄ GPI response was also observed with malaria-unexposed Caucasians. This indicates that immune responses against GPI or GPI-crossreactive carbohydrate antigens of non-malarial pathogens contribute to the overall anti-GPI antibody repertoire. Our data demonstrate, that results of serological analysis obtained with GPI isolated from *P. falciparum* parasites would critically depend on the ratio of Man₃ to Man₄ GPI in the antigen preparations used. In serological studies performed with purified GPI people who were not exposed to the malaria parasite were reported to completely lack anti-GPI antibodies (25). Epitope specificities of naturally occurring anti-GPI antibodies were also analysed with purified Man₄ GPI, Man₃ GPI and GPIs lacking a fatty acid substituent (26). In contrast to our results these studies showed a dual requirement of the glycan and lipid moieties of GPIs for antibody binding and, an absence of GPI specific antibodies in sera from healthy U.S. controls even when using Man₄ GPI coated ELISA plates. Assuming that the preparations used were pure and free from contamination, these findings could indicate that the coating of purified material in ELISA plates may result in a conformation of the GPI molecule where the access of anti-GPI antibodies directed only to the glycan moiety is hindered. Thus, while the microarray is limited in measuring immunoglobulins specific for glycans, the ELISA coated with purified GPIs may be unable to detect this sub-population of anti-GPI antibodies due to coating-related conformational problems or sterical hindrance by lipids.

Serum anti-GPI antibodies from healthy individuals living in the same malaria endemic area displayed highly diverse fine specificity patterns in synthetic-GPI microarray analysis, but the individual response patterns were strikingly stable. This finding is highly reminiscent to the observation, that antibody responses to polymorphic malaria protein antigens tend to have a fixed specificity that is unaffected by exposure to novel allelic types (27, 28). A comparable clonal imprinting mechanism on basis of an “original antigenic sin” (29) may also be operative, when the immune system is confronted with pathogens that express GPIs with different substitutions.

By comparing the levels and fine specificities of anti-GPI responses in healthy and diseased individuals the microarray-based approach identified glucosamine-inositol and glucosamine-inositol moieties coupled with one or two mannose sugars as relevant epitopes of anti-GPI antibodies that might be involved in protection against severe malaria. In our dualistic approach, the generated anti-GPI monoclonal antibodies (mAbs) with different antigen binding patterns resulted in a convergent finding: strong cross-reactivity with parasite-derived GPIs in IFA and Western blotting was only obtained with antibodies that also bound strongly to the glucosamine-inositol moieties without mannose residues. Thus, glucosamine-inositol moieties coupled with one or two mannose sugars may be the functional relevant epitopes accessible for protective anti-GPI antibodies, resulting in

effective anti-GPI neutralizing activity. These defined structural requirements for the binding of naturally and experimentally elicited anti-GPI antibodies should be considered in a rational design of GPI-based vaccine candidates. Finally, given the conservation in the core structure of GPIs across eukaryotes, it would be important for anti-GPI vaccination to avoid inducing autoimmune responses.

8.1.3 Lack of Reliable Surrogate Markers of Protection along the Malaria Vaccine Development Path

Malaria vaccine development is roughly divided in research and development, preclinical profiling and clinical development phases. The first two phases include measuring vaccine induced antibody titers and fine-specificities in preclinical models (e.g. by ELISA, IFA and Western blot) and correlating these qualities with some biologically relevant functional activity (verified in vitro and in vivo in murine models). Subsequently identified antigens targeted by potentially protective immune mechanisms are evaluated in non-human primate or other animal models for their safety, immunogenicity and efficacy. Even though some of the here described anti-GPI mAbs bound to parasite GPI and had accordingly to the serological array study the specificity (reactivity with GPI 1 to 3) predicted to be relevant for protection against severe disease, they failed to confer protection in functional assays; including in vitro parasite growth inhibition assays (GIAs), toxin neutralization assays (not shown) and passive protection experiments in animal infection models.

GIAs are currently the gold standard for in vitro activity of anti-malaria blood stage specific antibody responses and are explored to select antigens as candidates for preclinical development (30). GIAs with blood-stage parasites cultured in human red blood cells compare invasion and growth of parasites in the absence and presence of immunoglobulins. However, GIAs are very fragile test systems, hard to reproduce and therefore results have to be interpreted carefully. The correlation of in vitro growth inhibitory activities of antibodies with their potential protective capacity in vivo remains to be demonstrated.

TNF- α output by macrophages is used as a biochemical marker of malaria toxin activity in vitro. In toxin neutralization assays mouse macrophages or human peripheral blood monocytes are incubated with purified *P. falciparum* GPIs or total parasite lysates in the presence or absence of anti-GPI antibodies. Afterwards the culture supernatants are collected and TNF- α is measured by ELISA. A monoclonal antibody (23) to *P. falciparum* derived GPIs as well as sera from human patients infected with both *P. falciparum* und *P. vivax* (31) have been reported to neutralize the TNF- α -inducing activity of whole parasite extracts in vitro. However, like other investigators (20, J.B. de Souza et al., unpublished data) we could not confirm these findings in toxin-neutralisation

assays. The inconsistent results of this assay using extracted GPI suggest the existence of other parasite derived potent toxins in crude extracts or that not all anti-GPI antibodies are able to neutralize GPI activity in vitro. Analogous to the GIA, these assays are of limited predictive value.

The study of malaria vaccines is aided by the existence of experimental animal models, although rodent models are not available for infection with *P. falciparum*. The murine *P. berghei* ANKA (Antwerpen-Kasapa) strain is considered as a good pre-clinical model of severe disease (15). While many of the *P. falciparum* vaccine candidates are either not present or antigenically different in this species, we could evaluate GPI and the D13 protein in this model. Active and passive immunizations that targeted the conserved *PbD13* N^o-terminus failed to confer significant protection (data not shown). However, murine malaria is different from human malaria, and the mechanisms of immunity acting in such models may have no relevance for humans (32). Mice and rabbits represent good models to study the safety and immunogenicity of candidate vaccines but may fail to answer questions about protective efficacy (33).

The New World monkeys *Aotus* and *Saimiri* are the primate models used for preclinical trials of malaria vaccine efficacy, mainly because they can develop reproducible infections by both *P. falciparum* and *P. vivax* (34). Besides that these parasites have to be adapted to the artificial non-human hosts for successful infection, the primate animal models are difficult to access. While their usefulness in evaluating immunogenicity, safety and efficacy of potential candidates is well established and widely accepted, their predictive value for selection of protective vaccine candidates remains unclear (32).

This lack of reliable surrogate markers of protection poses a major problem for the screening and evaluation of malaria vaccine candidates. It is important to know that data supporting the current vaccine candidates rely on the above in vitro and in vivo approaches and on epidemiological associations between protection and antigen recognition by exposed individuals. Consequently vaccine candidates that show promise in a given model might fail in humans or worse, experimental results may lead to the rejection of vaccine candidates that would be effective in humans (32).

Once formulations and protocols have been defined and preclinical safety studies have been performed, candidates can be forwarded to human phase I clinical trials to evaluate safety, immunogenicity and the types of immune response elicited in non-immune volunteers living outside endemic areas. In phase IIa sporozoite challenge trials vaccine efficacy is evaluated as the ability to avoid or delay infection. Owing to ethical requirements, the trial end point is reached as soon as blood-stage parasites are detected. This is a limitation for evaluating blood-stage and anti-toxic vaccines, which may be effective in reducing parasite burden and toxin neutralisation but only after allowing a limited growth. Nonetheless, a recent study showed evidence of vaccine-induced blood-stage efficacy for the first time in a sporozoite challenge study (35). Volunteers vaccinated

with PEV3A, which included IRIV formulated peptides from both the pre-erythrocytic antigen CSP and the blood-stage antigen AMA-1, were not completely protected from malaria; but lower rates of parasite growth and the presence of morphologically abnormal parasites (crisis forms) were significantly observed in vaccinated volunteers.

The sporozoite vaccine RTS,S/AS02A and DNA-based heterologous prime-boost immunization strategies have shown some protection after experimental sporozoite challenge of non-immune volunteers but showed no or only limited efficacy in field trials (13, 14, 36, 37). The relevance of phase IIa challenge trials for predicting clinical efficacy thus has still to be demonstrated. Phase IIb trials target semi-immune volunteers from endemic areas and protection is assessed through the ability of vaccinated individuals to get no or less infections or to have longer intervals between infections, as compared with a control group (32). Therefore IIb trials are the generally accepted method of establishing proof of principle for blood-stage vaccines. Results on the efficacy of anti-toxin vaccine candidates would be obtained only very late in clinical development; in phase III trials the vaccine is delivered to children and infants of endemic regions and the end point may be a significant decrease in the incidence of clinical malaria or of severe disease in relation to a non-vaccinated population. Due to missing validated correlates of protection, the failures of the anti-GPI mAbs produced in this thesis in conferring protection in functional preclinical assays do not exclude synthetic GPI as a candidate for inclusion in an anti-disease vaccine.

8.1.4 Synthetic Polysaccharide Antigens need Enhancement for Immunogenicity in Infants

B cell activation by protein antigens requires both binding of the antigen by the B cell antigen receptor and interaction with antigen-specific helper T cells. With these thymus-dependent antigens, an immune response can occur shortly after birth, affinity maturation of the B cell response takes place, immunologic memory occurs and there is a heterogeneous immunoglobulin response (38). Some non-protein antigens stimulate B cells in the absence of linked recognition by peptide-specific helper T cells. Thymus-independent (TI) antigens elicit only limited isotype switching and do not induce memory B cells. These antigens can be divided into two classes, TI-1 or TI-2. TI-1 antigens, such as LPS, are potent B-cell mitogens, capable of non-specific, polyclonal activation of B cells. TI-2 antigens consist of molecules such as polysaccharides that have repetitive structures. Whereas TI-1 antigens can activate both immature and mature B cells, TI-2 antigens can activate only mature B cells (39). This might be why infants do not efficiently produce antibodies to polysaccharide antigens; most of their B cells are immature.

Therefore the generation of a vaccine-induced anti-GPI antibody response in children less than 5 years of age, which is the highest priority for vaccination in malaria-endemic areas, is critically dependent on the development of an antigen delivery platform that converts oligosaccharides into T cell dependent antigens. Experience with *Neisseria meningitidis*, *Haemophilus influenzae* type b and *Streptococcus pneumoniae* capsule polysaccharide conjugate vaccines has demonstrated that immunogenicity of carbohydrate antigens can be greatly improved by chemical conjugation to a protein carrier, especially in infants (40). For the development of an anti-toxic vaccine for malaria, it was proposed to conjugate synthetic GPI glycan to an immunogenic carrier protein, either of parasite (e.g. candidate blood-stage vaccine antigens, such as MSP-1) or bacterial (e.g. tetanus toxoid, diphtheria toxoid) origin (15). In addition, the immunogenicity has to be enhanced by delivery of the conjugates with an immunological adjuvant. With few exceptions, alum remains the sole adjuvant approved for human use in the majority of countries worldwide. This adjuvant has been used in practical vaccination for more than 60 years and is generally recognized as safe and as stimulator of Th2 immunity (41). Since alum represents only a sub-optimal adjuvant for recombinant proteins, synthetic peptides and polysaccharide antigens (41, 42), there is a major need for a safe and efficacious adjuvant capable of boosting cellular plus humoral immunity. Recently, immunopotentiating reconstituted influenza virosomes (IRIV) were evaluated as synthetic carbohydrate antigen delivery system (43). A leishmanial lipophosphoglycan-related synthetic tetrasaccharide was coupled to phosphatidylethanolamine or to hemagglutinin and embedded into virosomes. These vaccine formulations elicited in mice T cell dependent anti-glycan antibody responses that cross-reacted with native carbohydrate antigens expressed by leishmania cells. Experience with two licensed vaccines based on virosomes has shown that the technology is very safe and immunogenic even in children and infants (44-46). Therefore virosomes may be suitable as universal antigen delivery platform for an effective multi-valent anti-disease vaccine based on synthetic GPI in combination with synthetic blood-stage peptides or recombinant proteins.

8.1.5 mAbs serve as “All-Rounder” Tools in Infectious Disease Research, Therapy and Diagnostics

The antibodies produced in a natural immune response or after immunization are a mixture of molecules of different specificities and affinities. Heterogeneity results from the production of antibodies that bind to different epitopes on the immunizing antigen. The hybridoma technology has revolutionized the use of antibodies by providing a limitless supply of mAbs of a single and known specificity (47). Since each hybridoma is an immortalized clone derived from fusion of a myeloma

cell with a single B cell, all the antibody molecules it produces are identical in structure, including their antigen-binding site and isotype. The uniform characteristics of mAbs created an expanding role for these immunological reagents in vaccine research and development, therapy, and clinical diagnosis.

Within the framework of the present thesis, mAbs were generated to study *P. falciparum* and *B. anthracis* antigens, allowing the characterization of carbohydrate targets as candidates for diagnostic tests and vaccine design.

The ability to produce monoclonal antibodies allows backtracking from the desired antibody to its corresponding epitope. Immunization with the identified precise epitope may elicit similarly protective antibodies in the vaccinee. Such a reversed immunological approach is the first step in the rational design of a B cell epitope-based vaccine and was applied here with the generated anti-GPI mAbs. In addition, the anti-GPI mAbs may in future be used in combination with immunological, cell biological and biochemical methods to identify and study the GPI-anchored proteome in different life stages of *P. falciparum* and other *Apicomplexa* species. The newly identified GPI-anchored proteins are likely to include proteins involved in attachment and invasion of host cells or immune evasion mechanisms. They may comprise promising targets for parasite inhibitory antibodies and therefore represent new vaccine candidates.

Serum-therapy was one of the first effective means of treating infectious diseases but its use declined rapidly after the introduction of antimicrobial chemotherapy (48). Antibody-based therapies are currently undergoing a renaissance: there are more than 18 antibodies licensed for therapeutic use in the clinical settings of oncology, chronic inflammatory diseases, transplantation, infectious diseases and cardiovascular medicine (49). While polyclonal reagents suffer from a lack of reproducibility, mouse monoclonal antibodies used in humans can generate complications because of the host response to the foreign antibodies. In addition mouse antibodies may show only limited interactions with other elements of the human immune system resulting in inefficient effector functions and a short half-life (49). Humanization of murine antibodies is therefore a standard step in the development of therapeutic mAbs. Passive immunization of patients with severe malaria by administration of anti-toxin (GPI) antibodies is a thinkable therapeutic strategy, which could profit from the ongoing technological advances, including the synthesis of mouse–human chimeric antibodies, the immortalization of human peripheral B cells, direct cloning of variable genes into phage expression libraries and the creation of transgenic mice that produce human antibodies (50, 51). Passive anti-GPI antibody therapy may provide immediate immunity by neutralizing the toxic activities of parasite GPI and thereby reducing the over-stimulation of the immune system. This therapeutic proposal avoids the concern that inhibition of GPI-mediated early pro-inflammatory cytokine responses, which limit parasite growth, may favor rises in parasitaemia

in actively vaccinated people. In support of this antibody-based therapy, antibodies specific for bacterial super-antigens improved the clinical outcomes in streptococcal toxic shock syndrome, which is similar to the Th1 cytokine-dominated inflammatory response to severe malaria (52, 53). The time to develop an anti-GPI antibody therapy may be considerably shorter than that needed to produce a vaccine for infants. A clear limitation is that antibody drugs are expensive, which defines their use in developing countries. But high costs can be offset by unlikely development of resistance, since parasite GPI are invariant and essential for surviving.

Antibodies have provided the basis for specific and reproducible immuno-assays for the diagnosis of infectious diseases (54, 55). The uniform characteristics of mAbs have had a profound impact in the field of diagnostics by vastly improving the sensitivity and specificity of immuno-assays (56). Concerns with immunochemical techniques to detect *B. anthracis* endospores include problems with cross-reactivity and difficulties in obtaining species specific assays. mAbs generated against irradiated *B. anthracis* spores of the Ames and Sterne strains as well as against exosporium isolated from irradiated spores cross-reacted with *B. thuringiensis* and *B. cereus* spores (57). The majority of these mAbs were directed toward the protein component of the BclA glycoprotein. Because of specificity problems, as shown in this thesis, the development of an immuno-assay for detection of Anthrax spores based on anthrose is more difficult than anticipated (58). However, the mAbs specific for the synthetic anthrose-containing tetrasaccharide cross-reacted with spores of a panel of virulent *B. anthracis* strains covering a broad phylogenetic spectrum. The sensitivity of mAbs produced against the anthrose-rhamnose disaccharide (not shown) was even higher, since this mAbs detected also two unique Chadian isolates that represent a novel genetic lineage (A β) in the *B. anthracis* A cluster according to (59, 60). Biosafety containment requires an inactivation of spores containing probes by irradiation or thermal treatment. Interestingly the preparation of the *Bacillus* samples employed in this thesis, which included extensive boiling and filtration of loading-buffer denaturated spores, did not destruct the epitopes recognized by the carbohydrate specific antibodies. This conservation of the antigen was not self-evident, since it was demonstrated that inactivation methods could affect the sensitivity of nucleic acid- and antibody-based assays for the detection of *B. anthracis* endospores (61). ELISA or related solid phase immunoassay formats may represent the most useful forms of immunochemical method for the detection of *B. anthracis* spores, based on their simplicity and ability to analyze large numbers of samples at a time. The high-sensitivity quality of the anti-tetrasaccharide and disaccharide mAbs makes these immunological reagents ideal tools as capture antibodies for ELISA. Such highly sensitive spore detection ELISAs would be plagued by false-positive results but would afford a quick exclusion of negative samples from further analysis. Subsequent confirmatory expensive and time-consuming diagnostic tests with high specificity, such as real-time PCR or standard culture methods that focus

on the vegetative form, are therefore minimized. Improvements concerning the specificity of the proposed ELISA could be achieved in combining the sensitive anti-carbohydrate capture antibodies with detection antibodies of other specificities directed against *B. anthracis* spore antigens and/or unique markers of its closest relatives for exclusions. However, for a first screening of samples, which are suspected to contain *B. anthracis* spores, high sensitivity for the non-vegetative form is required to avoid false-negative results to guarantee civil protection in case of terrorist acts.

The efficacy of protective-antigen cell free anthrax vaccines is not satisfactory when compared with the live attenuated spore vaccine (62, 63). This could be because protective-antigen-based vaccines target toxemia and not bacteraemia. It is well established that full protection against anthrax requires a multifactorial immune response. *Bacillus* spores persist in the host for days after entrance into the body (64). Vaccination and passive immunization studies suggest that spore-specific Abs make a contribution to protection (65-67). The identity of these potentially protective Abs and their targets have yet to be determined (57). Exosporium antigens are likely to be involved in the interaction of spores with the infected host. The entry of the spore into macrophages is a critical step for *B. anthracis* infection. Spores are able to escape from phagolysosomes, and can germinate and multiply within the macrophage cytoplasm. The unique anthrose sugar could be included in a vaccine designed to elicit an immune response to *B. anthracis* spores. However, mice vaccinated with conjugated anthrose-containing tetrasaccharide were not protected following spore challenge, rather the opposite was observed (J. Frey, personnel communication). This indicates that the induced anti-carbohydrate antibody response might rather favour spore uptake by phagocytic cells after engaging Fc-receptors than inhibit the germination of spores in host macrophages.

8.2 Conclusions

In this thesis we have explored the potential of synthetically produced carbohydrate antigens of infectious agents as candidates for diagnostic tests and vaccine design. We presented a versatile screening method based on microarrays of synthetic carbohydrates that differentiates between malaria dependent and malaria independent adaptive immune responses to GPI. Human sera from malaria-exposed and malaria-free subjects exhibited differential binding patterns against two structurally distinct GPI glycans of *P. falciparum*, (EtNH₂)-Man₄-GlcNH₂-PI and (EtNH₂)-Man₃-GlcNH₂-PI. Array based serological studies supported the hypothesis that GPI is a target of clinical immunity and established a correlation between the presence of antibodies to certain structural elements of GPI and resistance to severe malaria. Adjuvanted synthetic oligosaccharide-protein conjugates promoted the generation of T cell dependent immune responses with different binding patterns against native carbohydrates, thereby representing ideal conditions for the generation of monoclonal antibodies. Anti-GPI mAbs emerged as a dualistic tool to experimentally investigate the role of GPI in malaria pathogenesis and to study the GPI-anchored proteome of *Apicomplexa* species. mAbs specific for anthrose-containing synthetic oligosaccharides generated in the framework of this thesis have potential as immuno-capturing components for a sensitive spore detection system. The consolidated findings obtained in this thesis demonstrated a great potential for synthetically produced oligosaccharides for bio-medical and research applications.

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**APPENDIX: PARASITE DENSITY-DEPENDENT REGULATION
OF A CCCH-TYPE ZINC FINGER PROTEIN IN
PLASMODIUM FALCIPARUM ASEXUAL BLOOD
STAGES**

Manuscript in preparation

PARASITE DENSITY-DEPENDENT REGULATION OF A CCCH-TYPE ZINC FINGER PROTEIN IN *PLASMODIUM FALCIPARUM* ASEXUAL BLOOD STAGES

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Keywords: CCCH-type zinc finger, *Plasmodium falciparum*, cell-to-cell communication, quantitative immuno-fluorescence analysis, post-transcriptional regulation

Abstract

A conserved protein of *Plasmodium falciparum*, designated parasite density controlled protein (PDCP; PF14_0652) with three tandemly arranged CCCH zinc finger motifs has been described by us. During screening of anti-PDCP mAb for parasite binding by indirect immunofluorescence analyses, we observed that the relative fluorescence signal strength in individual schizonts was associated positively with the parasite-density of cultures employed for staining. The relative PDCP expression was then assessed in high (10 %) versus low (2 %) parasite density cultures by quantitative laser scanning confocal microscopy. Experiments showed that the relative fluorescence intensity at least doubled in high density compared with low density parasite cultures. Interestingly, when 2 % cultures were grown for 48 h in the presence of cell-free parasite conditioned medium and then analyzed by IFA, the fluorescence signal obtained resembled that of 10 % cultures. Hence, soluble factor(s) in cultures of infected erythrocytes might mediate signal(s) resulting in up-regulation of PDCP expression. Therefore, a co-culture system was used whereby diffusible molecules of asexual blood stages can move freely between different parasite cultures separated by a semi-permeable membrane system. Co-culture of 2 % parasite cultures with 10 %, but not 2 % cultures or uninfected erythrocytes resulted in statistically significant increase of the mean of fluorescence intensity. No difference in PDCP mRNA levels was measured by Northern blot analysis and quantitative RT-PCR in the 2 % and 10 % parasite cultures indicative for post-transcriptional regulation of PfD13 expression. The inhibition of asexual parasite growth by anti-PDCP mAb in combination with evidence for regulation by parasite derived diffusible factors supports the hypothesis that PDCP might be involved in parasite growth regulation.

Introduction

The complex life cycle of *Plasmodium falciparum*, responsible for the most virulent form of malaria, requires specialized and regulated protein expression for the appropriate adaptation to both the mammalian host and insect vector. A search for transcription-associated proteins within the complete *P. falciparum* genome found that the parasite contained about a third of the number of proteins associated with the transcriptional process than present in genomes of free-living eukaryotes (Coulson et al., 2004) even though enzymes are found in equivalent abundance in malaria and yeast. Members of the CCCH-type zinc finger protein family are most prevalent within the *P. falciparum* genome when its abundance within the genome is normalized against the size of the genome providing further support for the hypothesis that protein levels in *P. falciparum* are controlled through post-transcriptional mechanisms (Coulson, Hall, and Ouzounis 2004). Post-transcriptional protein expression regulation has been described for genes involved in sexual differentiation, mitochondrial RNA processing and the stability of RNAs encoding for surface antigens (Le Roch et al., 2004). CCCH-type zinc finger proteins were first identified in differential screens for mRNAs that are rapidly induced following growth factor treatment of mammalian cells with the best-studied member of this class represented by the mouse protein tristetraprolin (Blackshear et al., 2005). Proteins containing zinc finger domains of the C-x8-C-x5-C-x3-H-type include proteins of eukaryotes involved in cell cycle or growth phase-related regulation and germline specification (Caro et al., 2005; Lai et al., 2000; Paterou et al., 2006; Shimada et al., 2006).

A conserved protein of *P. falciparum*, designated as parasite-density-controlled-protein (PDCP) (D13, PF14_0652) with three tandemly arranged CCCH zinc finger motifs has been described by us (Daubenberger et al., 2003). The protein was highly expressed during schizogony, declined rapidly in early ring stages and was undetectable in trophozoites assessed by immunofluorescence analysis (IFA) and Western blot analysis. Interestingly, inclusion of certain anti-PDCP mAbs targeting the CCCH-type zinc finger motif into asexual blood-stage cultures resulted in considerable inhibitions of parasite growth (Daubenberger et al., 2003).

Cell-to-cell communications via signal molecules play essential roles in synchronisation of gene expression and functional coordination. Since the concentration of some signal molecule(s) is a reflection of the population size, the term “quorum sensing” (QS) has been adopted to describe that accumulation of signal molecules enables individual cells to perceive when a minimum population unit, the “quorum”, is reached. QS is achieved through the production, release, and subsequent detection of and response to threshold concentrations of signal molecules and is well described in a wide variety of Gram positive and Gram negative bacteria (Bassler 2002). In recent

years evidence for QS has been also accumulating in eukaryotes. In *Histoplasma capsulatum* it has been shown that the yeast form produces some unique cell-wall polysaccharides (e.g. α -1,3-glucan) in a density-dependent fashion. If the fresh medium is supplemented with filtrate from a stationary-phase yeast culture, the cells continue to produce the α -1,3-glucan (Kugler et al., 2000). A similar density-dependent phenomenon has been described in *Ceratocystis ulmi*, the dimorphic fungus that causes Dutch Elm disease. *Ceratocystis ulmi* develop as budding yeast when inoculated at high density and as filamentous mycelia when inoculated at low density (Hornby et al., 2004). It has long been documented that *Candida albicans* hyphal formation is suppressed at high cell densities and by supernatants from stationary-phase *Candida albicans* cultures. The factors in these fungal cultures that represses hyphal development have been identified as farnesol and farnesoid acid (Hornby et al., 2001; Oh et al., 2001). In *Saccharomyces cerevisiae*, phenylethanol and tryptophol were identified as QS molecules mediating the induction of filamentous growth (Chen and Fink 2006). Proteins mediating QS in *Dictyostelium discoideum*, include a 80-kDa glycoprotein, a 450-kDa protein complex and countin 2 (Brock et al., 2003; Chen et al., 2005; Kolbinger et al., 2005). In this study we provide first time evidence that PDCP expression is density-dependent regulated in asexual blood stage cultures.

Materials and methods

Parasite culture. *P. falciparum* (K1 and 3D7) were maintained in human A+ erythrocytes at 5% hematocrit in RPMI 1640 supplemented with 25 mM HEPES, 24 mM NaHCO₃, 50 mg/l hypoxanthin and 0.5 % Albumax (complete culture medium). Highly synchronized ring stage parasite cultures were prepared by 5% (wt/vol) sorbitol treatment (twice at a time interval of 10 hours). The synchronized cultures were appropriately diluted into parallel cultures so that after a single 48 h cultivation period the cultures reached 2 % and 10 % parasite densities, respectively. Synchronization and parasite density were regularly confirmed by Giemsa staining.

Establishment of anti-PDCP mAb and indirect immuno-fluorescence analysis. A range of anti-PDCP mAb directed against the N-terminal 158 amino acids were established as described (Daubenberger et al., 2003). All hybridoma cultures were first screened in ELISA for specificity to the recombinant PDCP used for immunisation. Positive cultures were cloned twice, retested in ELISA for specific mAb production and expanded. Multi-test immuno-fluorescence microscopy slides (Flow Laboratories, Switzerland) were pre-treated with 0.01 % (w/v) poly-L-lysine (Sigma) for 30 min at room temperature and washed. Freshly isolated infected erythrocytes were washed and mixed with two volumes of a solution containing 4 % para-formaldehyde and 0.1 % Triton X-100. Droplets of 30 microl cell suspension were added to each well and incubated for 30 min at room temperature. Cells were blocked with blocking solution containing 100 mg/ml fatty acid-free bovine serum albumin in PBS. Cells were incubated with relevant mAb for 1 h. After several washing steps with blocking solution, cells were incubated with cyanine-dye-conjugated affinity-pure F(ab)₂ fragment goat anti-mouse Ig heavy chain antibodies (Jackson Immuno Research Laboratories, West Grove, Pa.) diluted in blocking solution containing 0.01 mg/ml Hoechst dye no. 33256 (Sigma). Antibody binding and DNA staining were assessed by fluorescence microscopy on a Leitz Dialux 20 fluorescence microscope and documented with a Leica DC 200 digital camera system. Magnification was 50 x and exposure time 0.4 sec and 1.2 sec for staining with Hoechst or antibody, respectively.

For relative quantification of the difference in PDCP expression by IFA, five images per slide from parasite cultures were recorded with the confocal laser scanning microscope (Leica TCS SP2 AOBS). Optical sections at interval of z μ m were recorded using a HCX-PL APO CS 40x 1.25 OIL immersion objective. All instrument settings (illumination, PMT, magnification, speed, averaging) were selected to collect fluorescence signal in the effective dynamic range of the PMT and kept constant throughout the whole experiment to allow for intensity comparison. Background signal was determined measuring mean intensity in cells incubated with the secondary Ab alone.

Images analysis was performed using Imaris Software (Bitplane, Switzerland). The mean Cy3 fluorescence emission signal intensity was quantified for each cell using the “spot function” of Imaris. Mean fluorescence intensity values obtained from cells stained with the identical mAb but grown under different parasite densities were compared statistically using the Students t-test using the STATA program.

Cell free conditioned medium preparation and parasite co-culture experiments.

Supernatant from *P. falciparum* (K1) high density parasite cultures (about 10 %) and uninfected RBC cultures cultivated similarly were collected. These supernatants were concentrated (25 fold) by Vivaspin 15R (Vivascience) with molecular weight cut offs of 30 kDa, dialyzed against complete culture medium and stored at +4 °C and are designated as cell free CM. Highly synchronized ring stage parasite cultures were treated with 1:100 and 1:1000 dilutions of the CM for 48 h. Cultures were harvested at late schizont stage and used for indirect immuno-fluorescence analysis and Northern blot analyses.

For the experimental co-culture system, parasites were synchronized by sorbitol treatment and then triplicate 0.4-mL test cultures were set up in Nunc (Roskilde, Denmark) 10-mm tissue culture insert wells with a 0.2- μ m pore Anopore membrane base (SPI Supplies, West Chester, PA) at 2 % parasitemia. Each group of three wells was supported within a 20-mL environmental culture with parasitemia of 2 %, 10 % and RBC only in a 140-mm diameter petri dish. Molecules were thus free to diffuse between environmental and test cultures, while the membrane contained the cells and parasites. Containment of merozoites was confirmed by absence of parasites within the RBC culture dish after co-culture with asexually replicating parasites. Co-culture cultures were harvested after 48 h for IFA and parasite growth was monitored by Giemsa staining (Dyer and Day 2003).

Northern blot analysis. Templates to prepare specific probes were generated by polymerase chain reaction (PCR) on K1 cDNA (Daubenberger et al., 2000) with PDCP specific primer pair 5-CAACAAAATGGTTTATGCCACAC-3 and 5-GAGAAGCGACAACATAATACAATA-3 yielding a 560 bp fragment. PCR products were purified using NucleoSpin columns (Macherey-Nagel, Oesingen, Switzerland) and labeled using [α -³²P]dCTP and the HighPrime system (Roche Diagnostics, Rotkreuz, Switzerland). Northern blotting was done essentially as described. Total RNA was prepared using TRIzol reagent (Invitrogen) and separated on 1 % agarose gels containing 5 mM guanidinium isothiocyanate. Equal loading (~5 μ g/lane) and quality of RNA were analysed visually under UV light and documented. The gel was then treated for 15 min in 0.05 M NaOH and in 10 \times SSC for 10 min; RNA was transferred to Hybond XL membranes (Amersham Biosciences, Dübendorf, Switzerland) over night by capillary transfer. Hybridization was carried out at 42°C in

UltraHyb (Ambion, Lugano, Switzerland) overnight. Filters were washed 2 x 5 min with 2 x SSC/0.1% SDS and 2 x 10 min with 0.1 x SSC/0.1% SDS at 42°C. Autoradiography was conducted at - 70°C by using a Transcreen HE enhancer and MS films (Eastman Kodak, Rochester, NY) (Daubenberger et al., 2003).

Quantitative real time-PCR. 20 ml cultures each with parasitemias of 2 % and 10 % were exposed to 0.05 % saponin in phosphate-buffered saline to lyse the erythrocytes. Parasites were pelleted and used for RNA extraction using the RNeasy mini kit (Qiagen). Total RNA extracted was eluted from the column in 50 ml of RNase free water. 10 ml was used for cDNA synthesis using the QuantiTect Reverse Transcription kit (Qiagen). The gene-specific oligo-nucleotide pairs used for PDCP (forward: 5- ATGGTTTATGCCACACTTTTGAGTG-3 and reverse: 5- GATCTAGTTGTATTATGTTGTCGCT-3) and 18S rRNA (SSU) of *P. falciparum* (forward: 5- CATTCGTATTTCAGATGTCAGAGGTG-3 and reverse: 5- CGTTCGTTATCGGAATTAACCAGAC-3) resulted in the amplification of PCR products of 565 and 482 base pairs, respectively (Nirmalan et al., 2002). To control for genomic DNA contamination, oligo-nucleotide pairs covering the intron sequence of glyceraldehyde-3-phosphate dehydrogenase (forward: 5- GAGCAGCCTTTGGAAGGAAAGATATC-3 and reverse: 5- TAAATGGGTCGTTAATAGCAACTACTTC-3) (Daubenberger et al., 2000) were employed. PCR was carried out in a volume of 100 µl and contained 5 U of Taq polymerase, 100 µM each dNTP, 200 ng of each primer pair, 2.5 mM MgCl₂. Cycling parameters were as follows: a pre-cycle at 95 °C for 5 min was followed by 30 cycles of 95 °C for 30 s, 30 s at 50 °C and 72 °C for 2 min with a final cycle of 72 °C for 10 min. Ten replicates of PCR reaction tubes for each amplification of 18S rRNA and PDCP were set up with cDNA of each culture and transferred into the PCR apparatus. Removal of individual PCR tubes was carried out at two-cycle intervals at the end of elongation phase without cycling disruption starting at cycle 10 up to cycle 30. Samples of each individual experiment were amplified in the same PCR apparatus and analysed on the same 1 % agarose gel. Molecular weight marker used was the 1 Kb plus DNA ladder (Invitrogen). Comparison of data among different samples and experiments was carried out by normalizing to the transcript levels of asexual 18S rRNA of *P. falciparum*. This procedure obviated the need to quantify total RNA samples before reverse transcription and to control for reverse transcriptase efficiency.

Results

Comparison of tandem CCCH-type zinc finger proteins in Apicomplexan parasites. We searched for PDCP orthologs using the public sequence data bases of twelve Apicomplexan parasites genomes including *Cryptosporidium hominis*, *C. parvum*, *P. berghei*, *P. chabaudi*, *P. falciparum*, *P. knowlesi*, *P. yoelii*, *P. vivax*, *Theileria annulata*, *T. parva*, *Toxoplasma gondii* and *Eimeria tenella*. The alignment of the deduced amino acid sequences of the N-terminal 145 amino acid residues of the PDCP orthologs is given in Figure 1. The three tandemly arranged CCCH-type zinc finger motifs are boxed with the conserved cysteine and histidine amino acid residues marked with stars. The first CCCH-type zinc finger motif follows the described C-x8-C-x5-C-x3-H-type in *E. tenella* and *T. gondii*, while the motif in the other organisms is arranged as C-x7-C-x6-C-x3-H. The second motif shows a C-x15-18-C-x5-C-x3-H spacing and the third motif the C-x7-8-x4-C-x3-H spacing pattern. In addition, many other residues are strictly conserved, including a RFRTK motif immediately preceding the first CCCH zinc finger motif. Within the 16 amino acid spacer located between the second and third zinc finger motif, 50% of the amino acid residues are conserved amongst these twelve apicomplexan species. Analyzing the nucleotide sequences of these PDCP orthologs for synonymous versus non-synonymous base exchanges indicated that a strong purifying selection pressure is operating resulting in high preservation of amino acid residues (<http://selecton.bioinfo.tau.ac.il>) (Doron-Faigenboim et al., 2005).

Conditioned medium of high density parasite cultures induces PDCP up-regulation.

While screening a panel of anti-PDCP mAb in IFA with synchronized schizont stages of *P. falciparum*, we observed inconsistent staining results. Therefore, schizonts of strains K1 and 3D7 were synchronized twice and then cultivated either at 2 % or 10 % parasite density, followed by parallel conducted IFA. Results with mAb D13.3 and D13.31 are shown in Figure 2A. Cells derived from cultures with 2 % and 10 % parasitemia are depicted in the first and forth row, respectively. Results of DNA control staining are given in the first and third column while the results of the staining with D13.3 and D13.31 are presented in the second and forth columns, respectively. Fluorescence intensities in individual schizonts at 10 % parasitemia cultures seemed to be higher compared with cells derived from 2 % cultures. This increased strength of the fluorescent signal was particularly evident in IFA using mAb D13.31 where in 2 % cultures, no fluorescence signal was detectable while cells derived from 10 % cultures were recognized (Figure 2A).

Next we addressed the question whether soluble factors accumulating in high-density parasite cultures might be responsible for changes of PDCP expression. Cell-free CM of high density (10 %) cultures of *P. falciparum* were sampled, concentrated and the 2 % parasite cultures

were treated with 1:100 and 1:1000 dilutions of the CM for 48 h starting with the ring stage. The IFA was conducted simultaneously with treated and untreated 2 % and 10 % cultures. In 2 % cultures exposed to 1:100 and 1:1000 diluted CM, enhanced fluorescence levels when compared to untreated 2 % cultures stained in parallel appeared (Figure 2A, second and third row). Supernatants of uninfected RBC suspensions did not influence PDCP expression levels (data not shown).

To quantify the observed differences in the immuno-fluorescence signal, schizonts were grown again at 2 % and 10 % densities and stained with mAb D13.44 and D13.26. Samples were analyzed by confocal laser scanning microscope followed by image analysis using the Imaris software for measurement of mean average fluorescence intensities. The results shown in Figure 2B demonstrated that a statistically significant difference in PDCP expression is seen between cells grown at 2 % and 10 % densities ($p < 0.001$).

For confirmation that a diffusible factor might mediate changes in PDCP expression, an experimental co-culture system was used where molecules of asexual blood stage cultures move freely between parasite cultures but are un-permeable for cells. Co-culture of 2 % parasite cultures with 10 % but not with 2 % cultures resulted in increased fluorescence signals (Figure 3A). As shown in Figure 3B, parasite cultures grown in vicinity of 10 % cultures had significantly higher mean fluorescence intensities compared to identical cultures grown in the neighbourhood of 2 % cultures ($p < 0.001$) (Figure 3B).

To exclude the possibility that PDCP up-regulation resulted merely from cellular responses to nutrients depletion or waste accumulation in the high density cultures, parasite growth was assessed by $^3\text{[H]}$ -Hypoxanthin incorporations assays. No difference in 2 % cultures grown in the presence or absence of CM preparations used in the IFA experiments was observed (data not shown). It is well known that in standard cultures of malaria parasites growth is limited to parasitemia of 10 % – 15 % of erythrocytes. Hence at 2 % and 10 %, the parasite cultures have not reached yet their maximum density and no signs of cell degradation could be detected by Giemsa staining of culture smears (data not shown).

Evidence for post-transcriptional regulation of PDCP expression. To relate the increase in PDCP protein expression to changes in mRNA levels, Northern blot analysis was conducted with identical samples shown in Figure 2A. Total RNA was extracted from cultures and equal amounts of total RNA was loaded from each sample as evidenced by the ethidium-bromide stained agarose gels before transfer to Hybond-N membranes. The unique signals obtained after hybridisation with PDCP-specific radioactively labelled probes were comparable between the lanes loaded with mRNA derived from 2 %, 10% and schizont cultures treated with 1:100 dilution of CM, respectively (Figure 3A). As a second approach for analysis of changes in PDCP specific mRNA a

quantitative RT-PCR was conducted. This quantitative RT-PCR is based on the observation that the abundance of a given template sequence shows a reverse linear relationship with the cycle number at which the amplification product is first detected above the baseline level (the threshold cycle). The RT-PCR amplification products of the 18S rRNA from cDNAs derived from 2 % and 10 % cultures became visible at cycles 20 and 16, respectively. The RT-PCR products of PDGP amplified from the identical cDNAs appeared at cycles 26 and 24, respectively (Figure 3B). Hence, in relation to the threshold detection of 18S rRNA, significant changes in PDGP mRNA levels in 10 % versus 2 % cultures was not found.

Discussion

In the current study we provide to our knowledge the first report of parasite density-dependent regulation of a CCCH-type zinc finger protein in *P. falciparum* asexual blood stage. CCCH-type zinc finger proteins (<http://www.ebi.ac.uk/interpro>; IPR000571) that possess the characteristic C-x8-10-C-x5-C-x3-H sequence of the prototype CCCH-type zinc finger domain were first identified in differential screens for mRNA that is rapidly induced following growth factor treatment of mammalian cells. The best-studied member of this protein class is represented by the mouse protein tristetraprolin (TTP) (Blackshear 2002). TTP can shuttle between the nucleus and cytoplasm and the best-documented function of the protein is its ability to bind to AU-rich elements in certain mRNA molecules and to destabilize those mRNA, apparently by initiating a deadenylation process (Lai, Carballo, Thorn, Kennington, and Blackshear 2000; Phillips et al., 2002). Knockout mice lacking TTP show chronically over-expressed tumour necrosis alpha protein and granulocyte macrophage-colony stimulating factor levels due to stabilization their encoding mRNAs in macrophages derived from TTP-deficient mice (Taylor et al., 1996). In *Caenorhabditis elegans*, CCCH-type zinc finger proteins have been shown to be involved in the differentiation of germ cells during embryonic development and function via temporal and spatial regulation of maternal mRNA at levels of transcription, mRNA processing, RNA localization and translation (Mathesius et al., 2003; Shimada, Yokosawa, and Kawahara 2006; Shimada et al., 2002).

In Trypanosomes, the paucity of known transcriptional control and the poly-cistronic organization of the genome make post-transcriptional mechanisms the most important level for gene regulation (Clayton 2002). RNAi mediated knockdown and genetic ablation of one of these zinc finger proteins, TbZFP1, severely compromised the differentiation from bloodstream to pro-cyclic forms in *Trypanosoma brucei* (Hendriks et al., 2001; Hendriks and Matthews 2005). The authors speculate that TbZFP1 influences the differentiation through stabilizing or destabilizing mRNA with AU-rich elements or through modulating their cellular location or capacity for translation. A family of proteins characterized by their single zinc finger motif C-x7-8-C-x5-Cx3-H and small size (<150 amino acids with x representing any amino acid residue) has been demonstrated in *Trypanosoma cruzi* with TcZFP1 binding selectively to poly(C)homo-polymers *in vitro* (Morking et al., 2004). Interestingly, TcZFP1 and TcZFP2 interact with each other via the WW domain of TcZFP2 (Caro et al., 2005).

The co-regulation of PDCP mRNA expression with mRNA of proteins involved in host cell invasion (guilt by association (Le Roch et al., 2003)), in combination with results of the high throughput yeast two hybrid-system analysis of *P. falciparum* (LaCount et al., 2005) indicate that PDCP is involved in biological processes during schizont development, merozoite release or host

cell invasion. Orthologue sequences of PDCP with the conserved three tandemly arranged CCCH-type zinc finger motives have been so far exclusively found in the genome sequences of the Apicomplexa *C. hominis*, *C. parvum*, *E. tenella*, *P. berghei*, *P. chabaudi*, *P. knowlesi*, *P. vivax*, *P. yoelii*, *T. annulata*, *T. parva* and *T. gondii*. Thus, members of this sub-family of CCCH-type zinc finger proteins might mediate conserved biological processes shared between these organisms.

The observed PDCP up-regulation could be explained as the result of diffusible, so far unknown factor(s) accumulating in high-density parasite cultures. This assumption was supported by the observations that the effect of CM on PDCP expression was concentration dependent and results of the two-chamber co-cultivation experiments. In *P. falciparum*, several independent observations and theoretical considerations support the possible involvement of diffusible molecules in cell-to-cell communication. Asexual bloodstream parasites responded to host-derived diffusible molecules (Lingnau et al., 1993; Maswoswe et al., 1985) and secreted a somatostatin-like hormone (Pan et al., 1987). Diffusible factors from log-phase asexual blood stages of *P. falciparum* stimulated their own growth and replication. The presence of the parasite was essential for growth regulation and the process was density-dependent (Dyer and Day 2003). Soluble factor(s) of unknown biochemical nature in parasite-CM stimulated gameto-cytogenesis *in vitro* (Coulson, Hall, and Ouzounis 2004). Monitoring of experimental human *P. falciparum* infections indicated the existence of a density-dependent stimulation mechanism of parasite growth in humans since parasite growth accelerated from logarithmic (days 1 – 4) to an above logarithmic rate (days 6 – 8) in malaria naïve subjects inoculated with an asexual blood-stage *P. falciparum* stabilate (Cheng et al., 1997). In studies of the longitudinal dynamics of multiple Plasmodium spp. infections in semi-immune, asymptomatic children in Papua New Guinea, the total parasite density of Plasmodium spp. oscillated around a distinct threshold distinct from the fever threshold. The authors concluded that malaria parasitemia might be controlled in a density-dependent manner and that additionally cross-species mechanisms of parasite density regulation might exist (Bruce et al., 2000b; Snounou 2004). Since it is well known that acquired immunity to Plasmodium infections is both species- and genotype-specific (Bruce et al., 2000a), a different mechanism could be responsible for the observed species-transcending nature of the density-dependent regulation. Whether the stable parasite density is driven by a host-derived, innate immune mechanism like nitric oxide production or by parasite-derived mechanisms remained so far unclear (Kwiatkowski 1995). Uncontrolled proliferation of *P. falciparum* asexual blood stages will result in host death, sometimes before the parasite can be transferred to the insect vector. Hence, one could hypothesize that the parasite might not rely exclusively on the host immune system to control its growth. The asexually proliferating parasite could regulate its density directly by (i) the rate of cell division; (ii) the rate of RBC invasion and (iii) the rate of cell death (Deonte and Becker 2004). Assuming that parasite derived

mechanisms limit the density of the parasites, then the questions arise like (i) what might be the physiological substrate for this cell-to-cell communication, (ii) does it resemble the QS mechanisms described in other organisms and (iii) which proteins are regulated by these factor(s)?

In recent years, there have been several reports of QS-like phenomena in fungal species. Intriguingly, each of these cases involved a morphological transition from a filamentous, mycelial form to a yeast form, or vice versa. Whether this apparent theme represents the most common application of QS in eukaryotes, or the limited search for QS in eukaryotes, is not known. The characterisation of components of CM mediating PDGP up-regulation may lead to elucidation of possible mechanisms of cell-to-cell communication and development regulation in *P. falciparum*.

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Figures

Fig. 1. Multiple amino acid sequence alignment of N-terminus of PDCP orthologues with the three zinc finger motifs (CCCH type) boxed. Sequences were aligned using CLUSTAL W (1.83) and included *P. chabaudi* (PC000922.02.0), *P. yoelii* (MALPY00805), *P. berghei* (AY293729), *P. knowlesi* (pkn2318a12.pk1), *P. vivax* (Pv117045), *Theileria parva* (TP01_0814), *T. annulata* (TA05800), *Cryptosporidium parvum* (EAK88000.1) and *C. hominis* (AAEL01000292). (*) conserved residues and positions of the conserved cysteine and histidine amino acid residues are boxed. Databases searched included <http://www.sanger.ac.uk>, <http://www.tigr.org>, <http://www.ncbi.nlm.nih.gov> and <http://plasmodb.org> (August 2007).

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<i>Cryptosporidium hominis</i>	MACPRLSDNDLCRFRTKP	CRRSKQMGCDGFIGITRCQYSH	NIYWPRRCPPFYLSNQSTIRYIPVL 63
<i>Cryptosporidium parvum</i>	MACPRLSDNDLCRFRTKP	CRRSKQMGCDGFIGITRCQYSH	NIYWPRRCPPFYLSNQSTIRYIPVL 63
<i>Plasmodium berghei</i>	MVYATLLSEEDLSRFRTKQ	CKRLLNGGCNFGLDRCQYSH	NEFWNRRCPFYLSDSSFIRYITVM 63
<i>Plasmodium yoelii</i>	MVYATLLSEEDLSRFRTKQ	CKRLLNGGCNFGLDRCQYSH	NEFWNRRCPFYLSDSSFIRYITVM 63
<i>Plasmodium chabaudi</i>	MVYATLLSEEDLSRFRTKQ	CKRLLNGGCNFGLDRCQYSH	NEFWNRRCPFYLSDSSFIRYITVM 63
<i>Plasmodium falciparum</i>	MVYATLLSEEDLSRFRTKQ	CKRLLNGGCNFGLDRCQYSH	NEFWNRRCPFYLSDSSFIRYITVM 63
<i>Plasmodium knowlesi</i>	MVYATLLSEEDLSRFRTKQ	CKRLLNGGCNFGIDRCQYSH	NEFWNRRCPFYLSDSSFIRYITIM 63
<i>Plasmodium vivax</i>	MVYATLLSEEDLSRFRTKQ	CKRLLNGGCNFGIDRCQYSH	NEFWNRRCPFYLSDSSFIRYITIM 63
<i>Theileria annulata</i>	MPILSEEDLERFRTKV	CTLASSMKCDFGVERCNYSH	NLYWARRCPFYLRDSSILRYIPQC 60
<i>Theileria parva</i>	MPILSEEDLERFRTKV	CTLASSMKCDFGVERCNYSH	NLYWARRCPFYLRDSSILRYIPQC 60
		* * * *	
<i>Cryptosporidium hominis</i>	CPDIIIKEDESSI-SHCNRGGGCPFAH	SYEEINYHPLMYKTKI	CEQFQRGDCNTYYCHLIH 123
<i>Cryptosporidium parvum</i>	CPDIIIKEDESSI-SHCNRGGGCPFAH	SYEEINYHPLMYKTKI	CEQFQRGDCNTYYCHLIH 123
<i>Plasmodium berghei</i>	CPNIETKSDGSIN-SLCLRGGECPPFAH	STEEILYHPLFYKTKR	CEDYKKGSCNTYYCPYVH 123
<i>Plasmodium yoelii</i>	CPNIETKSDGSIN-SLCLRGGECPPFAH	STEEILYHPLFYKTKR	CEDYKKGSCNTYYCPYVH 123
<i>Plasmodium chabaudi</i>	CPNIETKSDGSIN-SVCLRGGECPPFAH	STEEILYHPLFYKTKR	CEDYKKGSCNTYYCPYVH 123
<i>Plasmodium falciparum</i>	CPDVETRGDGSIN-SLCLRGGECPPFAH	STEEILYHPLFYKTKR	CEDYKKGSCNTYYCPPFIH 123
<i>Plasmodium knowlesi</i>	CPDVETKSDGSIN-SLCLRGGECPPFAH	SABEILYHPLYKTKR	CEDYKKGSCNTYYCPPFIH 123
<i>Plasmodium vivax</i>	CPDVETKSDGSIN-SLCLRGGECPPFAH	SABEILYHPLYKTKR	CEDYKKGSCNTYYCPPFIH 123
<i>Theileria annulata</i>	CPDVELGEGTTVIRNSCPRGNNCSFAH	SYEEIHYHPLVYKTEV	CKDYRLGKCKTYCHLVH 121
<i>Theileria parva</i>	CPDVELGEGTTVIRNSCPRGNNCSFAH	SYEEIHYHPLVYKTEV	CKDYRLGKCKTYCHLVH 121

Fig. 2A. Co-cultivation of 2 % *P. falciparum* cultures with cell-free conditioned medium results in PDCP up-regulation. Synchronized schizonts grown to 2 % and 10 % densities were analyzed by IFA with mAb D13.3 and D13.31. In parallel, 2 % cultures that were incubated for 48 h (starting from ring stages) with 1:100 and 1:1000 final dilutions of conditioned medium were stained and analysed. In the negative control, the secondary antibody only was applied to 10% parasite cultures. Parasite DNA staining with Hoechst 33258 is given in blue.

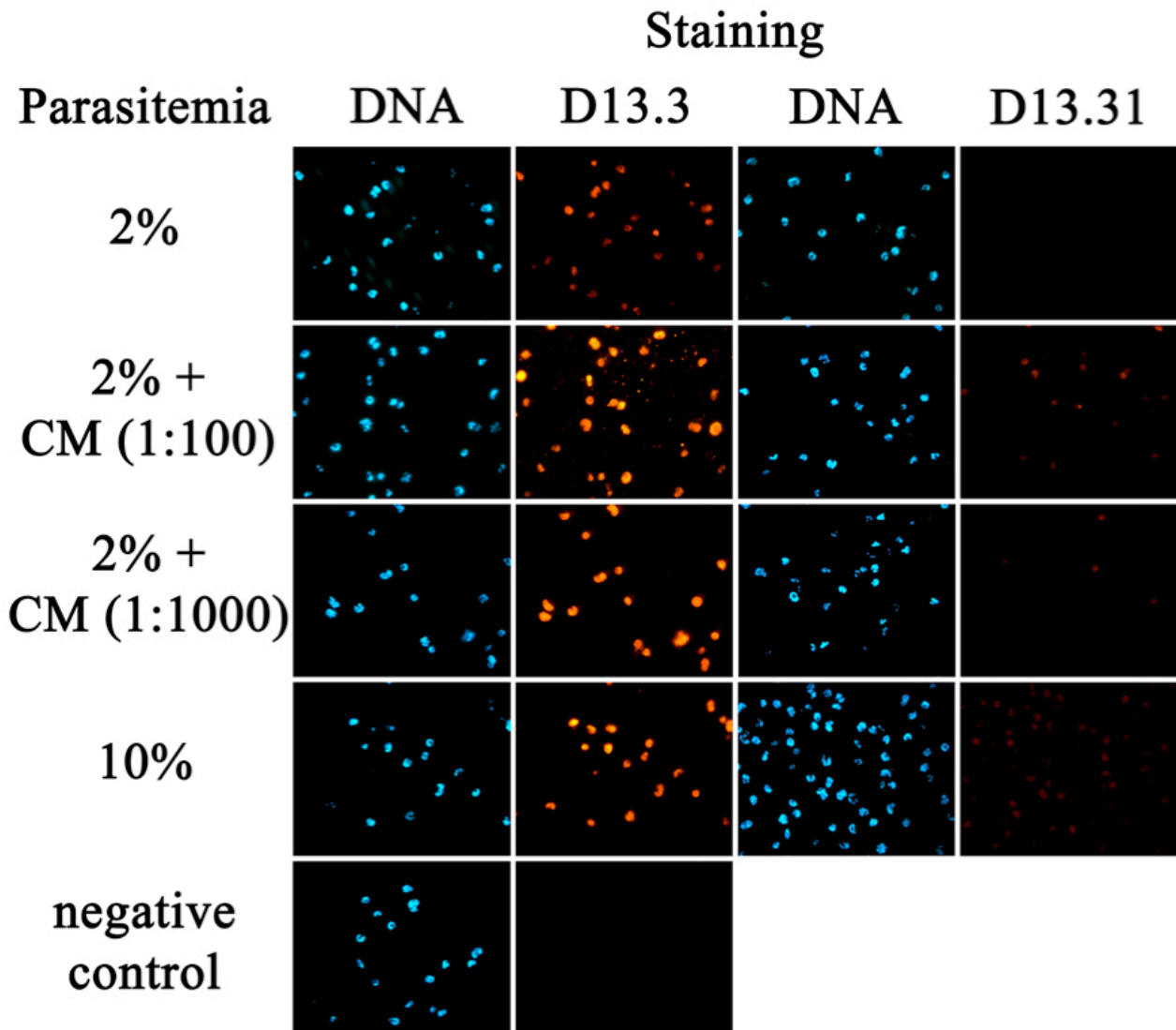


Fig. 2B. Relative quantification of PDCP expression after cultivation of schizonts at different densities. Synchronized schizonts grown to 2 % (white bars) and 10 % (grey bars) densities were stained for PDCP expression using mAb D13.26 and D13.44. Cells were analyzed by laser scanning confocal microscopy as described in materials and methods. The average mean intensity values after subtracting the background signals (mean fluorescence intensity of cells incubated with secondary antibody only) for each cell population are shown. The difference in expression level of PDCP assessed with both mAb was statistically significant ($p < 0.001$).

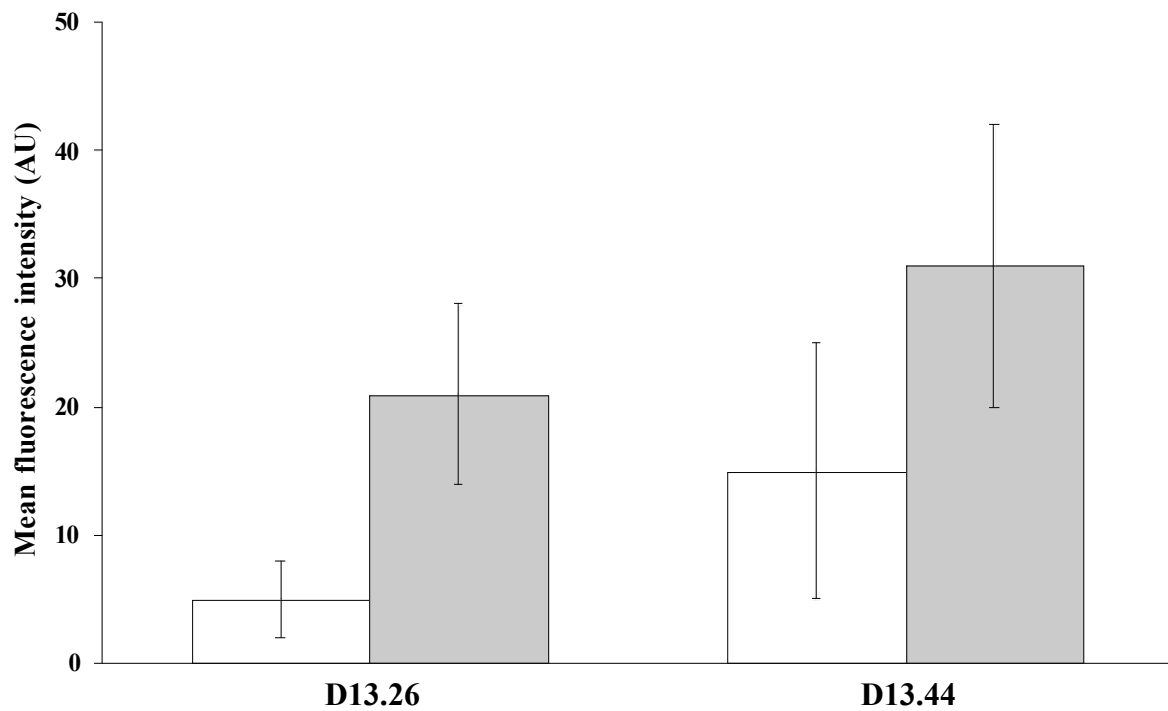


Fig. 3A. Two-chamber co-cultivation of *P. falciparum* asexual blood stages. Synchronized 2 % *P. falciparum* cultures were either cultivated with 2 % parasites (I) in a co-cultivation chamber or (II) with 10 % parasite cultures for 48 h starting at ring stages. Cells were removed and stained with Hoechst 33258 (binding to parasite DNA) or subjected to IFA using mAb D13.44.

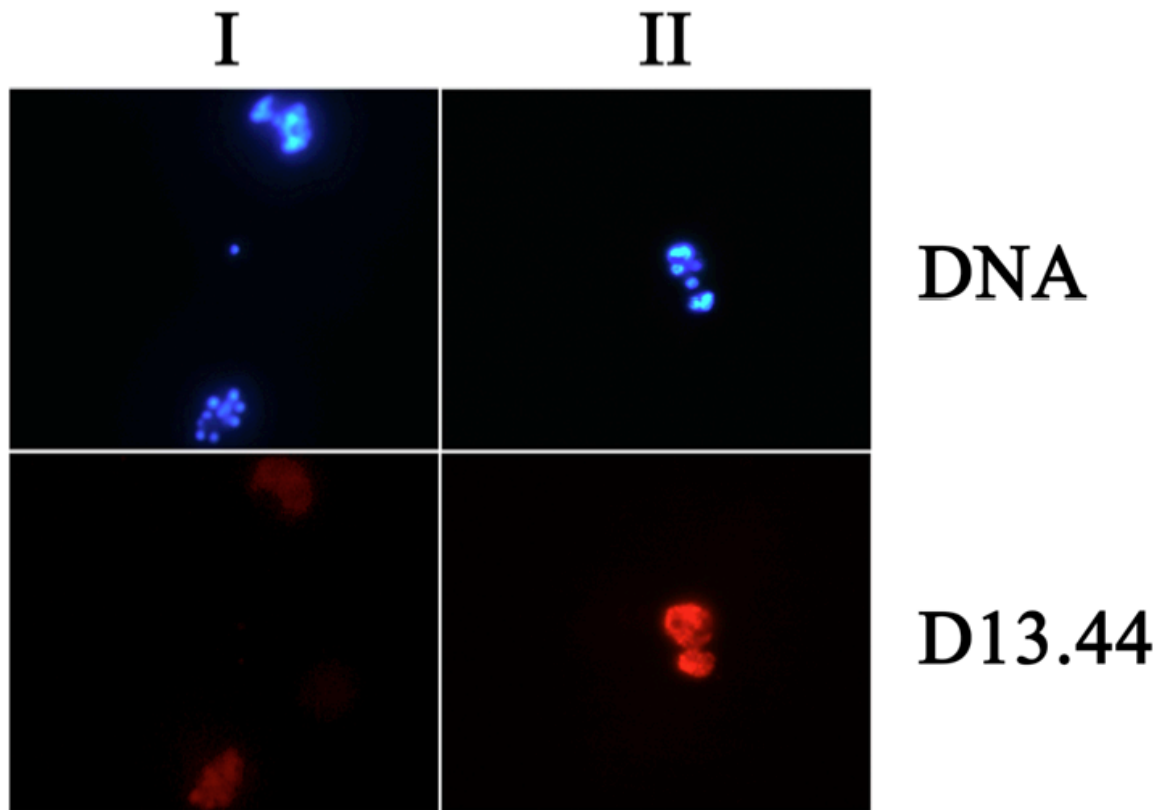


Fig. 3B. Relative quantification of PDCP after trans-well co-cultivation experiments.

Synchronized schizonts were co-cultivated in a trans-well chamber at 2 % with parasite populations grown to 2 % (white bars) or 10 % densities (grey bars) at the end of the cultivation period. Cells were removed from culture and stained with mAb D13.26 and D13.44. Schizonts were analyzed by laser scanning confocal microscopy and the average mean intensity values after subtracting the background signals (mean fluorescence intensity of cells incubated with secondary antibody only) for each cell population are shown. The difference in expression level of PDCP assessed with both mAb was statistically significant ($p < 0.001$).

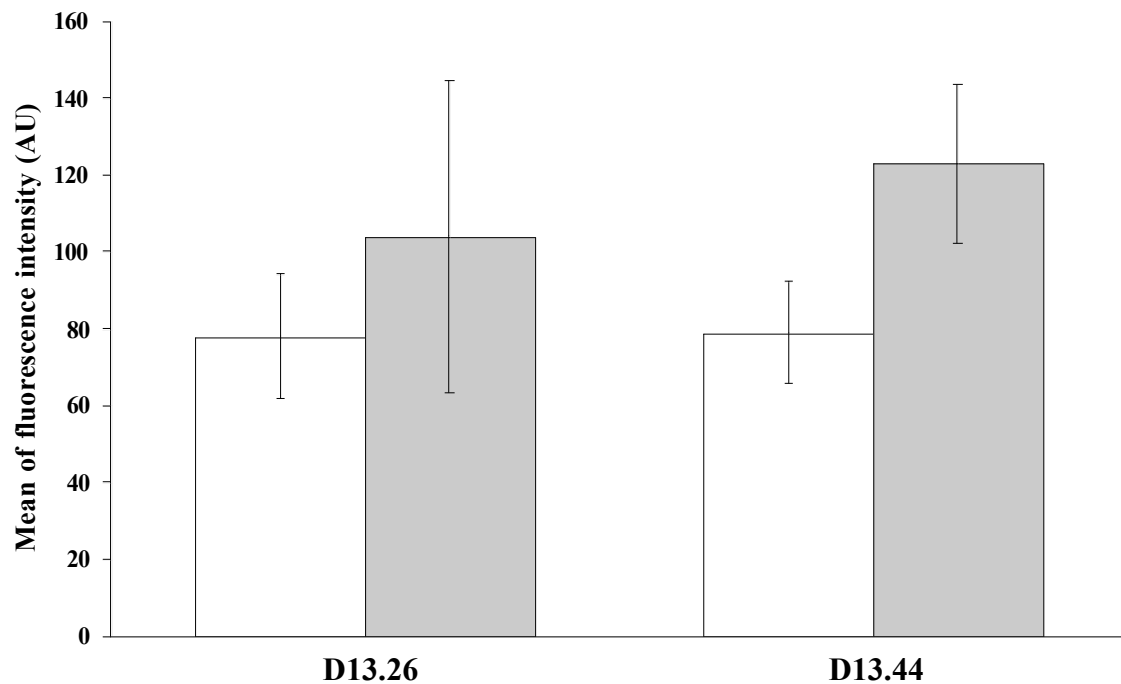


Fig. 4A. Analysis of PDCP mRNA expression levels by Northern blot analysis. Cultures given in Figure 2A were used for RNA extraction. Untreated 2 % cultures, cultures treated with 1:100 dilutions of CM and 10 % parasite cultures are given in (A), (B) and (C), respectively. Equal amounts of extracted total RNA was separated by agarose gel electrophoresis (II) and then blotted onto nylon membrane and hybridized to [α - 32 P]-dCTP-labelled probe corresponding to the 5'-terminal 422-bp fragment of PDCP (I). Size markers are given on the left.

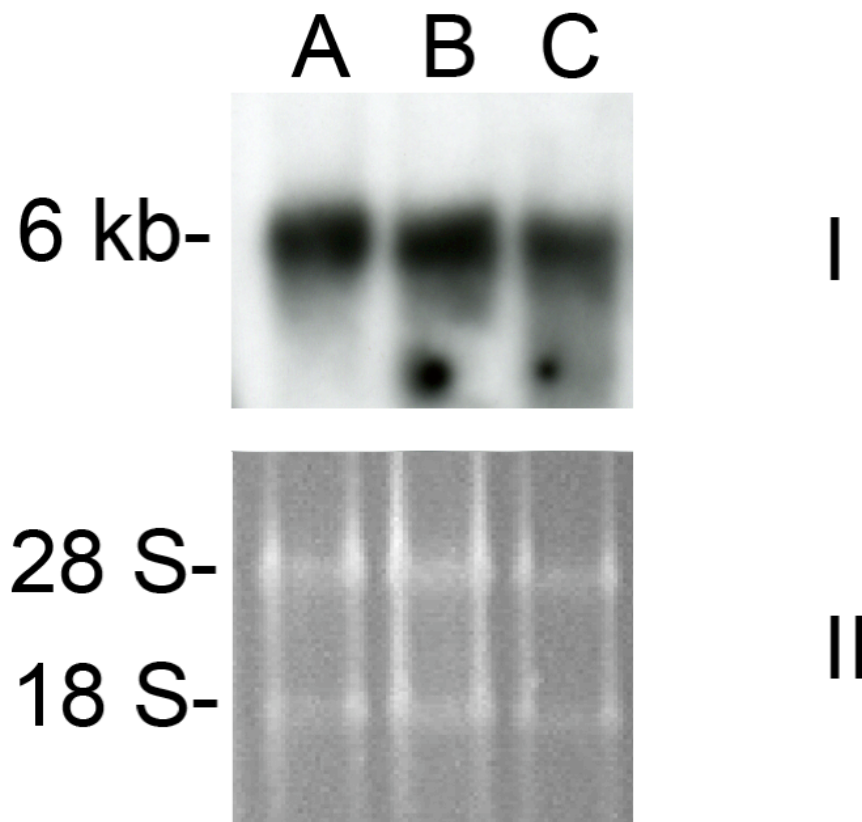
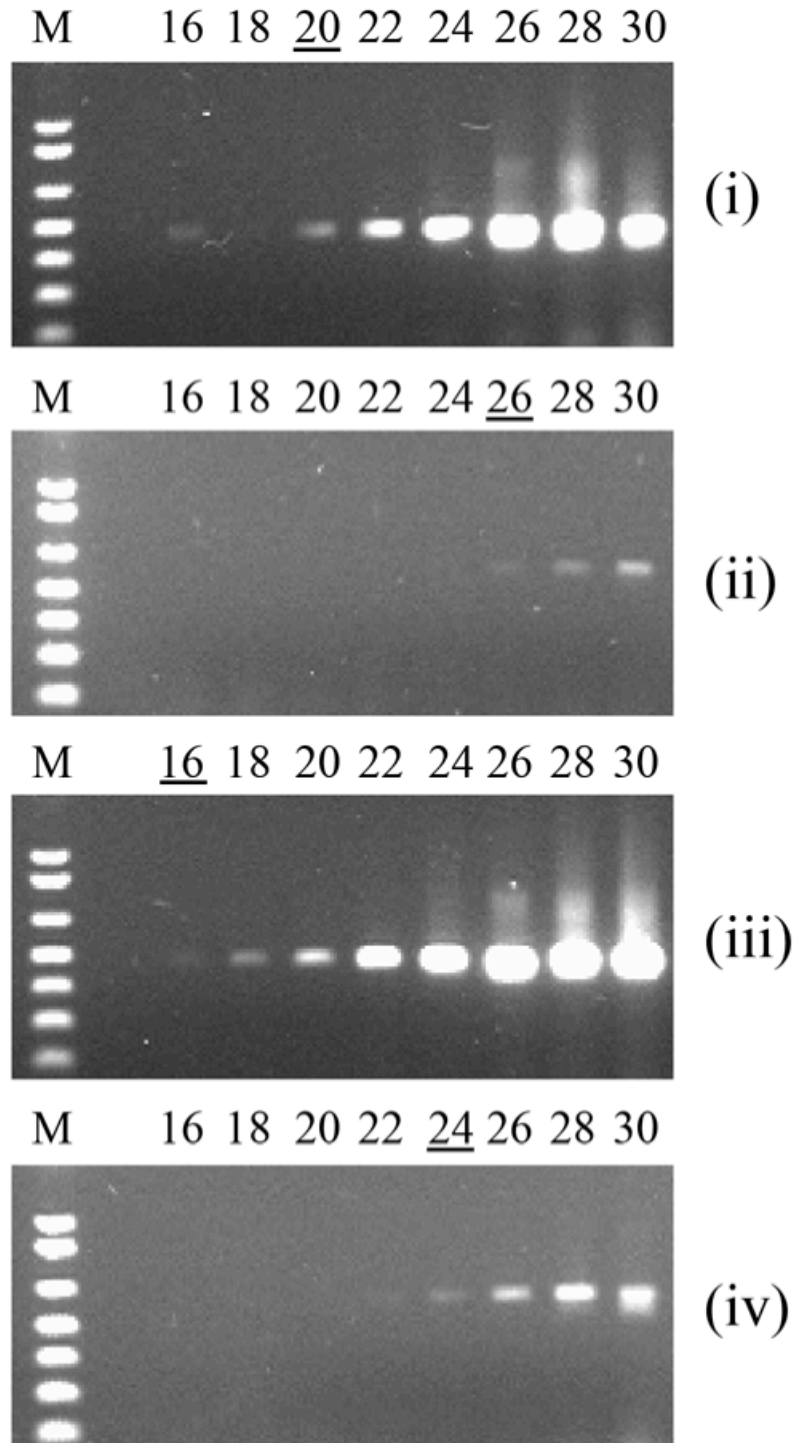


Fig. 4B. Quantitative RT-PCR analyses of PDCP in 2 % and 10 % parasite cultures. RT-PCR products derived from 2 % (i), (ii) and 10 % (iii), (iv) *P. falciparum* cultures are shown. In (i) and (iii) 18S rRNA was amplified yielding an unique product of 482 base pairs, while in (ii) and (iv) PDCP was amplified as a product of 565 base pairs. Lane numbers correspond to the cycle number at which the PCR tube was removed from the PCR machine. The underlined numeral marks the cycle number at which the RT-PCR products became observable in the 1 % agarose gel stained with ethidium bromide. Molecular weight marker (M) is given on the left.



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Education	
2000-2004	University of Basel, Switzerland, field of study: Organismic Biology
2003-2004	Diploma in Molecular Immunology: Protective activities of antibodies against the Malaria antigen D13. Molecular Immunology, Swiss Tropical Institute, Basel, Switzerland. Advisors: PD. Dr. Claudia Daubenberger and Prof. Dr. Gerd Pluschke
2004-2008	PhD in microbiology: Use of Synthetic Carbohydrates as Vaccine Components and Biomedical Research Tools. Molecular Immunology, Swiss Tropical Institute, Basel, Switzerland. Advisor: Prof. Dr. Gerd Pluschke
2005	Introductory Course in Laboratory Animal Science, Institut für Labortierkunde, University of Zürich, Switzerland
2007	Visiting scientist at the Biomedical Primate Research Centre (BPRC), Prof. Alan Thomas, Rijswijk, The Netherlands
Presentations	
2005	PhD student meeting of the Swiss Society of Tropical Medicine and Parasitology, Ascona, Switzerland. Talk: Comparative genomics of apicomplexan parasites: Characterisation of parasite-growth inhibiting antibodies recognizing D13-orthologues in <i>Plasmodium</i> and <i>Theileria</i> spp.
2006	Joint meeting of the Royal Society of Tropical Medicine & Hygiene and the Swiss Society of Tropical Medicine and Parasitology, Basel, Switzerland Talk: Synthetic carbohydrate microarrays as new tool to investigate human immune responses to <i>Plasmodium falciparum</i> GPI and for the production of monoclonal anti-GPI antibodies

- 2007 Meeting of the Swiss immunology PhD students at Schloss Wolfsberg, Switzerland
Talk: Synthetic carbohydrate microarrays as new tool to investigate human immune responses to *Plasmodium falciparum* GPI and for the production of monoclonal anti-GPI antibodies
- 2008 Swiss Tropical Institute Research Seminar, Basel, Switzerland
Talk: Use of synthetic carbohydrates as vaccine components and biomedical research tools

Publications

- 2006 **Tamborrini M, Werz DB, Frey J, Pluschke G, Seeberger PH.** Anti-carbohydrate antibodies for the detection of anthrax spores. *Angew Chem Int Ed Engl.* 2006, 45, 6581-6582.
- 2008 **Kamena F, Tamborrini M, Liu X, Kwon YU, Thompson F, Pluschke G, Seeberger PH.** Synthetic GPI array to study antitoxic malaria response. *Nature Chem. Biol.* 2008, 4, 238-240.
- 2008 **Tamborrini M, Kamena F, Seeberger PH, Pluschke G.** Functional activities of monoclonal antibodies raised against synthetic *Plasmodium falciparum* Glycosylphosphatidylinositol glycans. Manuscript in preparation.
- 2008 **Tamborrini M, Kamena F, Seeberger PH, Pluschke G.** Synthetic glycosylphosphatidylinositol microarray reveal differential antibody levels and fine-specificities in children with mild and severe malaria. Manuscript in preparation.
- 2008 **Tamborrini M, Siegrist S, Bohrmann B, Oroszlan-Szovik K, Pluschke G, Daubenberger CA.** Parasite density-dependent regulation of a CCCH-type zinc finger protein in *Plasmodium falciparum* asexual blood stages. Manuscript in preparation.
- 2009 **Tamborrini M, Oberli AM, Werz DB, Schürch N, Frey J, Seeberger PH, Pluschke G.** Immuno-detection of Anthrose Containing Tetrasaccharide in the Exosporium of *Bacillus anthracis* and *Bacillus cereus* Strains. *J Appl Microbiol.* 2009, (Epub ahead of print)

Patents

- 2006 European Patent No. 06116359.8- Detection of *Bacillus anthracis* and vaccine against *B. anthracis* infections

Laboratory Skills

- Microarray-based and basic immunoassays (ELISA, Western blotting, IFA)
- PCR set-up
- FACS analysis
- Generation of monoclonal antibodies with hybridoma technology
- Isolation of immunoglobulin class switch variants of hybridomas by sequential sublining
- *Plasmodium falciparum* culture and in vitro growth inhibition assay
- Laboratory animal expertise and in vivo protection assays

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