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Microarray analysis of the human antibody response to synthetic *Cryptosporidium* glycopeptides

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ABSTRACT

Glycoproteins expressed by *Cryptosporidium parvum* are immunogenic in infected individuals but the nature of the epitopes recognised in *C. parvum* glycoproteins is poorly understood. Since a known immunodominant antigen of *Cryptosporidium*, the 17 kDa glycoprotein, has previously been shown to bind to lectins that recognise the Tn antigen (GalNAcα1-Ser/Thr-R), a large number of glycopeptides with different Tn valency and presentation were prepared. In addition, glycopeptides were synthesised based on a 40 kDa cryptosporidial antigen, a polymorphic surface glycoprotein with varying numbers of serine residues, to determine the reactivity with sera from *C. parvum*-infected humans. These glycopeptides and non-glycosylated peptides were used to generate a glycopeptide microarray to allow screening of sera from *C. parvum*-infected individuals for the presence of IgM and IgG antibodies. IgG but not IgM in sera from *C. parvum*-infected individuals bound to multivalent Tn antigen epitopes presented on glycopeptides, suggesting that glycoproteins from *C. parvum* that contain the Tn antigen induce immune responses upon infection. In addition, molecular differences in glycosylated peptides (e.g. substituting Ser for Thr) as well as the site of glycosylation had a pronounced effect on reactivity. Lastly, pooled sera from individuals infected with either *Toxoplasma* or *Plasmodium* were also tested against the modified *Cryptosporidium* peptides and some sera showed specific binding to glycopeptide epitopes. These studies reveal that specific anti-glycopeptide antibodies that recognise the Tn antigen may be useful diagnostically and in defining the roles of parasite glycoconjugates in infections.

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1. Introduction

Cryptosporidium parvum is a protozoan parasite that infects the epithelial cells of the small intestine causing diarrheal illness in humans. It has a worldwide distribution and is considered an emerging zoonosis with reservoirs in cattle, domestic animals and in faecally-contaminated environments (Davies and Chalmers, 2009). Transmission via the faecal–oral route results from the ingestion of *Cryptosporidium* oocysts through the consumption of contaminated food or water (drinking and recreational water) or through direct person-to-person or animal-to-person contact. In immunocompetent individuals, cryptosporidiosis is usually an acute self-limiting gastroenteritis, resolving within 2–3 weeks. Symptoms include diarrhoea (3–6 stools per day), weight loss, fever and fatigue. In immunocompromised persons, however, infection is associated with more persistent symptoms and serious illness, especially in those with HIV, primary immunodeficiency or those undergoing solid-organ transplantation (Leitch and He, 2012). Drug treatment is very limited; nitazoxanide has demonstrated some efficacy in immunocompetent individuals and is approved for children under the age of 12 years. However, it has not proven effective for treatment of immunocompromised persons (Huang et al., 2004; Fox and Saravolatz, 2005). Additionally, no vaccines are currently available for use against *C. parvum*.

While sequencing of the *Cryptosporidium* genome has facilitated the characterisation of the protein components of these antigens, little is known about the glycan moieties in parasite glycoconjugates. Several *C. parvum* membrane proteins/antigens that are associated with invasion and/or protection include various mucin-like proteins (Boulter-Bitzer et al., 2007). Two *Cryptosporidium* immunodominant antigens, Cp17 and Cp40, are major surface glycoproteins (Cevallos et al., 2000; Priest et al., 2000; Strong et al.,

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2000) that carry multiple GalNAcα1-Ser/Thr-R epitopes, here termed O-linked GalNAc glycans. They also represent potential vaccine targets (Benitez et al., 2009; Manque et al., 2011). These antigens are encoded by a single gene, Cp40/17, and subsequently cleaved into the Cp40 and Cp17 proteins. Within the Cp40 antigen there is a hypervariable region with predicted O-glycosylation sites following the conserved polyserine domain (Leav et al., 2002). The *C. parvum* 17 kDa antigen glycosylphosphatidylinositol (GPI) anchor is composed of a very basic GPI anchor core comprised of Manα1-2Manα1-6Manα1-4-glucosamine (Priest et al., 2006), but there are also minor GPI phospholipids that are recognised by serum antibodies in infected humans (Priest et al., 2003).

In the present study, antibody responses were evaluated against GalNAc-containing glycopeptides using defined synthetic glycan microarrays as a mechanism for characterising and identifying glycans that may be important antigenic components. Additionally, we synthesised glycopeptides of the Cp15/17 and Cp40/45 antigens, including novel synthetic poly-Tn sequences, based on the observations that these two immunodominant antigens contain potential Tn (GalNAcα-Ser/Thr-R) antigen sites (Gut and Nelson, 1999) and that sera from infected individuals reacted to glycosylated antigens (Moss et al., 1994). Our results show that such defined glycopeptide microarrays can help to characterise the effect of glycosylation on immunoreactivity and could be useful in future developments of diagnostic assays and understanding of host–parasite interactions.

2. Materials and methods

2.1. Synthesis of peptides

Cp17 and Cp40 Tn glycopeptides were synthesised by standard Fmoc-protected solid phase peptide synthesis (Liu et al., 2005; Bolscher et al., 2010; Borgert et al., 2012). The glycosylated Ser or Thr residues (Sussex Research Laboratories, Ontario, Canada) were incorporated into the growing peptide chain in the same manner as the non-glycosylated sequences, creating defined site-specific glycosylation, meaning that the glycosylated residues could be added at specific places in the sequence rather than indiscriminately enzymatically glycosylating a whole peptide. This allowed us to determine which glycosylation site(s) is important for binding. A unique feature of the Cp40 peptides is the repeating poly-Ser-GalNAc residues, where multiple glycosylated serine residues are found in sequence in the native protein and which were therefore synthesised (Strong et al., 2000). These long chains of Ser-GalNAc were difficult to synthesise and required longer and repeated reaction times compared with standard Fmoc synthesis, and have not been previously reported to be synthesised in the literature. However, peptides were made with up to 23 Ser-GalNAc residues in succession to compare the differential binding to these varying glycosylated repeats. Cp17 #1–7 contained different combinations of glycosylated Ser/Thr residues, and Cp17 #9 and 10 had the Ser and Thr residues switched from the native sequence to determine whether the GalNAc linkage to the Ser or Thr is critical for binding. Cp40 #1, 3 and 5 contained 7, 16 and 23 repeating units of Ser-GalNAc, respectively. As controls, the non-glycosylated sequences were also produced and printed (Cp17 #8 and 10, Cp40 #2, 4 and 6).

2.2. Glycan and glycopeptide arrays

To identify glycans and glycopeptides that are recognised by antibodies in serum of *Cryptosporidium*-infected people compared with uninfected people, multiple sets of glycan and glycopeptide microarrays were used. The mammalian glycan microarray available from the Consortium for Functional Glycomics (CFG) (http://www.functionalglycomics.org/static/index.shtml) was comprised of over 500 glycans. A mannose-6-phosphate array containing high mannose-type N-glycans of defined structure containing zero, one or two mannose-6-phosphate-GlcNAc phosphodiester or mannose-6-phosphate phosphomonoester residues was utilised (Bohnsack et al., 2009; Song et al., 2009a). A Tn antigen (GalNAc-Ser/Thr) glycopeptide array was constructed with a variety of glycopeptides and control peptides, containing sequences of various origins that are O-glycosylated at Ser/Thr sites (Borgert et al., 2012). The Tn glycopeptide array was expanded to contain the new Cp17- and Cp40-specific sequences (see Table 1) as well as recombinant Cp17 and Cp23 proteins (printed at 200 μg/ml) (Priest et al., 1999, 2000).

Glycans containing a reducing alkyl amine or homogeneous peptides and glycopeptides containing N-terminal amino groups or lysine residues were directly printed by covalent attachment to microarray slides containing N-hydroxysuccinimide derivatised surfaces (Song et al., 2009a,b). For peptide/glycopeptide microarray studies, all materials were printed at 100 μM concentration (except as noted above), as previously described (Song et al., 2008, 2009b) using precision printing by a piezoelectric printing approach in which 1/3 nl of samples were deposited in ~100 micron-sized spots. The slides were washed, dried, and stored indefinitely in anhydrous conditions.

2.3. Human sera

For *Cryptosporidium* sera, individual, anonymised sera were used for this study. Positive samples (P; $n = 10$) were collected during two cryptosporidiosis outbreaks from donors who met the outbreak case definition but who were not stool-confirmed. Five additional positive sera (G) were included from patients who reacted strongly to the *C. parvum* glycosylinositol phospholipids (GIPLs) (Priest et al., 2003). These samples were collected during three different cryptosporidiosis outbreaks and included two donors who were stool-confirmed. All positive samples (collectively called P samples) were assayed using the 'gold standard' western blot assay (Moss et al., 1998) and were determined to be strongly reactive. Negative samples (N; $n = 5$) were from western blot negative donors. Additionally, pools of high titer serum samples (10 serum samples per pool) from individuals acutely infected with *Plasmodium falciparum* or *Toxoplasma gondii* were tested. All serum samples were diluted 1:100 before analysis and IgM and IgG binding assays were quantitated separately, based on previous CFG array data using serum and preli-

Table 1
Synthesis of *Cryptosporidium parvum* Cp17 and Cp40 peptides. Cryptosporidial peptides Cp17 (Cp17 #1–10) and Cp40 (Cp40 #1–6) were synthesised with different Tn valency (Cp17) and length of repeating serine residues (Cp40) to determine how these groups affect sera antibody binding.

Peptide number	Sequence, (*) denotes Tn antigen on S or T
Cp17_#1	H-ETS*EAAAT*VDLFAFT*LDGGK-NH2
Cp17_#2	H-ETSEAAAT*VDLFAFT*LDGGK-NH2
Cp17_#3	H-ETS*EAAATVDLFAFT*LDGGK-NH2
Cp17_#4	H-ETSEAAATVDLFAFT*LDGGK-NH2
Cp17_#5	H-ETS*EAAAT*VDLFAFTLDGGK-NH2
Cp17_#6	H-ETSEAAAT*VDLFAFTLDGGK-NH2
Cp17_#7	H-ETS*EAAATVDLFAFTLDGGK-NH2
Cp17_#8	H-ETSEAAATVDLFAFTLDGGK-NH2
Cp17_#9	H-ETT*EAAAS*VDLFAFS*LDGGK-NH2
Cp17_#10	H-ETTEAAASVDLFAFSLDGGK-NH2
Cp40_#1	H-DVPVEGSS* (7)TSTVAPANK-NH2
Cp40_#2	H-DVPVEGSS(7)TSTVAPANK-NH2
Cp40_#3	H-DVPVEGSS* (16)TSTVAPANK-NH2
Cp40_#4	H-DVPVEGSS(16)TSTVAPANK-NH2
Cp40_#5	H-DVPVEGSS* (23)TSTVAPANK-NH2
Cp40_#6	H-DVPVEGSS(23)TSTVAPANK-NH2