Experimental Parasitology 130 (2012) 314–320

Contents lists available at SciVerse ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Terminal galactosylation of glycoconjugates in *Plasmodium falciparum* asexual blood stages and *Trypanosoma brucei* bloodstream trypomastigotes

Ranjan Ramasamy [a,b,*], Mark C. Field [a]

[a] Department of Pathology, University of Cambridge, Cambridge, UK
[b] Institute of Health Sciences, Universiti Brunei Darussalam, Gadong, Brunei Darussalam

ARTICLE INFO

Article history:
Received 10 April 2011
Received in revised form 29 September 2011
Accepted 15 February 2012
Available online 3 March 2012

Keywords:
Bandeirea (Griffonia) simplicifolia B4 lectin 1
Galactosylation
Glycoconjugate
Plasmodium falciparum
Trypanosoma brucei

ABSTRACT

There is definitive biochemical evidence for the presence of terminal α-galactosyl residues (α-gal) in the N-linked oligosaccharides and glycophosphatidylinositol anchors (GPI anchors) of the variant surface glycoprotein of *Trypanosoma brucei* bloodstream trypomastigotes. Indirect evidence also exists for α-gal in *Plasmodium falciparum* asexual blood stage glycoproteins and glycolipids. The occurrence of α-gal in glycoproteins and glycolipids of *T. brucei* bloodstream trypomastigotes and *P. falciparum* late asexual blood stages was investigated by the binding of α-gal-specific *Bandeirea simplicifolia* B4 lectin 1 (BSB4), incorporation of [^{3}H]galactose from UDP-[^{3}H]galactose into glycoproteins and glycolipids in microsomes in vitro, and bioinformatic searches for galactosyl-transferase coding sequences. The findings confirm the presence of α-gal in a spectrum of *T. brucei* bloodstream trypomastigote glycoproteins and glycolipids and indicate its relative absence from *P. falciparum* asexual blood stage glycoconjugates.

1. Introduction

Malaria had an annual incidence of 247 million cases worldwide in 2008 with nearly one million deaths, much of it in sub-Saharan Africa. *Plasmodium falciparum* causes cerebral malaria and most of the worldwide malaria mortality (World Health Organisation, 2010a). African trypanosomiasis caused mainly by *Trypanosoma brucei gambiense* is a debilitating and often fatal disease with an estimated 30,000 cases in sub-Saharan Africa (World Health Organisation, 2010b). A better understanding of parasite biochemistry and the host immune response to infection can generate new methods for reducing mortality and morbidity in malaria and trypanosomiasis.

Biochemical studies have established the presence of non-reducing, terminal α-linked galactosyl residues (α-gal) in the glycosylphosphatidylinositol (GPI) membrane anchors (Ferguson et al., 1988) and N-linked oligosaccharides (Zamze et al., 1991) of the *T. brucei* variant surface glycoproteins (VSG), and the GPI anchor of its transferrin receptor (Mehlert and Ferguson, 2007). Biochemical studies also indicate the presence of lactosyl ceramide (Uemura et al., 2006) and glycosylinositol phospholipids (GIPLs) carrying α-gal (Mayor et al., 1992; Guther et al., 2003) in *T. brucei* bloodstream trypomastigotes. Alpha-gal is also found on the free GIPLs of promastigotes of some *Leishmania* species and O-linked oligosaccharides of *Trypanosoma cruzi* cell-derived trypomastigote mucins (McConville and Ferguson, 1993; Almeida et al., 1994). The molecules that contain α-gal are postulated to play important roles in the biology/immunobiology of these trypanosomatid parasites (McConville and Ferguson, 1993; Zamze et al., 1991; Almeida et al., 1994; Ferguson, 1999; Buscaglia et al., 2006).

While studies from several laboratories have suggested the presence of α-gal in the asexual blood stages of *P. falciparum,* the glycoconjugates displaying them were not definitively characterised (Ramasamy and Reese, 1985, 1986; Jakobsen et al., 1987; Ravindran et al., 1988; Ramasamy and Rajakaruna, 1997; Mya et al., 2002). Other data suggests that *P. falciparum* and another apicomplexan parasite, *Toxoplasma gondii,* synthesise mono- and digalactosyl diglycerides (MGDG and DGDG, respectively), as well as monogalactosylceramide based on chromatographic migration properties and reaction with a specific antibody against DGDG (Marechal et al., 2002). DGDG contains a terminal α-1-6-linked galactose attached to the β-galactose of MGDG. MGDG and DGDG are the major glycolipids of chloroplast membranes (Harwood, 1980) and could therefore be derived from the evolutionarily related *P. falciparum* apicoplast (Foth and McFadden, 2003). Biochemical characterisation indicates that GPI-anchors of *P. falciparum* lack α-gal (Gerold et al., 1994; Gowda et al., 1997), in contrast to those of bloodstream *T. brucei* trypomastigotes (Ferguson et al., 1988). *P. falciparum* GPI-anchor fragments activate inflammatory responses (Tachado et al., 1996; Krishnegowda et al., 2005) and elicit human antibodies (Naik

* Corresponding author at: Institute of Health Sciences, Universiti Brunei Darussalam, Jalan Tungku Link, Gadong BE 1410, Brunei Darussalam.
E-mail address: ranjan.ramasamy@ubd.edu.bn (R. Ramasamy).

0014-4894/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2012.02.017

et al., 2000). In a *P. berghei* mouse model, immunisation with the cognate GPI-glycan protected against pathology and anti-GPI antibodies neutralised tumour necrosis factor production by *P. falciparum* in vitro (Schofield et al., 2002). Furthermore, a role for NKT cells in GPI anchor-induced cytokine production in malaria has been reported (Schofield et al., 1999; Hansen et al., 2003).

In contrast to the GPI membrane anchors of *P. falciparum*, little is known about the biological properties and functions of the glycosphingolipids and glycoglycerolipids reportedly synthesised by the parasite (Marechal et al., 2002; Gerold and Schwartz, 2001; Couto et al., 2004; Landoni et al., 2007). Poor IgM antibody responses to *P. falciparum* glycolipids associated with hyper-reactive malarial splenomegaly in an endemic area of Venezuela suggests a role for glycolipids in malaria pathology (Vivas et al., 2008). Considerable synthesis of new membranes as well as remodelling of the erythrocyte and parasite surface membranes takes place during the development of *P. falciparum* inside erythrocytes, and it is possible that such lipids have important roles in these processes. Glycolipids such as α-galactosyl ceramide are potentially capable of influencing innate immune responses as well as antibody and T cell-mediated adaptive immune responses (Tupin et al., 2007). Hence the possible presence of α-gal containing glycolipids and glycoproteins in *P. falciparum* is relevant to understanding the biochemistry and immunology of falciparum malaria.

We therefore investigated the presence of galactosylated glycoconjugates in *P. falciparum* late asexual blood stages compared to *T. brucei* bloodstream trypomastigotes by lectin binding and in vitro labelling of microsomal preparations with UDP-[^{3}H]galactose.

2. Methods and materials

2.1. Parasite culture

The 3D7 isolate of *P. falciparum* cultured essentially according to standard procedures (Trager and Jensen, 1976) was obtained from the National Institute of Medical Research, London and the Wellcome-Sanger Institute, Hinxton (gifts of A.A. Holder, M. Grainger and S. Campino). Late trophozoites and schizonts were separated from uninfected red cells and more immature stages by magnetic field separation or Percoll density gradient centrifugation to yield >80% late stage parasites. Bloodstream trypomastigotes of *T. brucei* strain Lister 427, variant 221 was cultured according to standard procedures (Chung et al., 2008; Mayor et al., 1992).

2.2. Binding of FITC-labelled Bandeirea (Griffonia) simplicifolia I isolectin B4 (BSB4) to whole parasites

This was done essentially as described (Ramasamy and Reese, 1986). Smears of freshly cultured and washed parasites on glass microscope slides were fixed in 90% acetone/10% methanol (v/v) at −20°C for 10 min and reacted with 100 μg/ml of FITC-conjugated BSB4 lectin (Sigma) for 2 h at 26°C. The slides were then washed and examined with UV illumination in a Nikon Eclipse E600 epifluorescence microscope at 1000× magnification. Images were captured using a Hamamatsu CCD camera driven with Metamorph software (Molecular Devices).

2.3. Characterisation of parasite proteins binding to BSB4

BSB4 (Sigma) was chemically coupled to CNBr-activated Sepharose beads (Pharmacia) according to the manufacturer's instructions. Parasite lysates were prepared by lysing 50 μl parasites or unparasitized red cells with 500 μl of 0.5% Triton X-100 in TBS, pH 7.2 containing 0.1 mM $CaCl_2$, 100 μg/ml of leupeptin and pepstatin and 0.1 mM TLCK. Centrifuged lysates were then incubated with 50 μl of BSB4-Sepharose beads with mixing for 2 h at 26°C. The beads were subsequently washed and eluted with Laemmli sample buffer. Aliquots of the eluates were analysed by SDS–PAGE followed by silver staining of the gel or Western transfer to PVDF membrane and probing with biotin-BSB4 lectin (Sigma). For probing, the membranes were reacted with biotin-BSB4 at 5 μg/ml, followed by streptavidin-peroxidase (Sigma) and development with the chemiluminescence substrate luminol following standard procedures.

2.4. Preparation of glycolipids from parasites

This was done essentially as described (Christie, 1993). 0.2 ml of parasites or control unparasitized red cells were homogenised in ice cold 3.8 ml of chloroform/methanol (CM 2:1 v/v). The extract was centrifuged and the supernatant subject to Folch washing (Christie, 1993). The pellet was extracted with chloroform/methanol/water (CMW 10:10:3 v/v/v) and pooled with the Folch-washed CM 2:1 extract. The pooled extract was filtered through nylon mesh, dried and dissolved in n-butanol. The n-butanol solution was extracted with water, dried and the glycolipids then redissolved in CM 2:1 v/v and stored at −80 °C.

2.5. Characterisation of P. falciparum glycolipids reacting with BSB4 and immune human sera

Glycolipids from parasites or unparasitized red cells were subject to silica gel TLC using CMW 65:25:4 or CMW 10:10:3 v/v/v as solvent. TLC plates with separated glycolipids were blocked in 3% BSA in tris-buffered saline and then reacted with biotin-labelled BSB4 at 2 μg/ml followed by streptavidin-peroxidase (Sigma) at 2 μg/ml and colour development with diaminobenzidine according to standard procedures. Separated glycolipids were also reacted with pooled Ugandan human sera known to react with *P. falciparum* asexual blood stages by ELISA (Naus et al., 2003) and control pooled sera from Europeans not exposed to malaria (gift of D. Dunne). The plates were washed and then sequentially treated with peroxidase-conjugated anti-human Ig antibodies and substrate.

2.6. Preparation of microsomes from parasites and labelling with UDP-[^{3}H]galactose

This was done by modification of a described procedure (Masterson et al., 1990). Freshly cultured parasites were washed in PBS and resuspended at 10^9 cells per ml in ice-cold water containing 1 μg/ml each of leupeptin and pepstatin and 0.1 mM TLCK for 5 min. Lysis was checked microscopically. An equal volume of 100 mM HEPES buffer pH 7.4, containing 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM TLCK, 1 μg/ml each of leupeptin and pepstatin and 20% glycerol was then added and 1 ml aliquots rapidly frozen in dry ice and stored at −80 °C.

To prepare microsomes, the lysate was rapidly thawed at 37°C, and to 0.5 ml aliquots was added 1 ml buffer containing 50 mM HEPES pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 0.1 mM TLCK, and 1 μg/ml each of leupeptin and pepstatin. The lysate was centrifuged at 20,000 g for 10 min at 4°C in an Eppendorf 5417 microfuge. The pelleted microsomes were washed twice in the same buffer and resuspended in 0.25 ml of the same buffer plus 5 mM $MnCl_2$, 1 mM ATP and 0.5 mM DTT and the mix transferred to a 1.5 ml Eppendorf tube containing dried 1.8 MBq of UDP-[^{3}H]galactose (specific activity 0.16–1.5 TBq/mmol, Radiochemical centre, Amersham or American Radiolabeled Chemicals Inc., St Louis, MO). The microsomes were then incubated with mixing at 37°C for 30 min. Five microliter aliquots were removed for protein analysis and the remaining microsomes extracted with 1.7 ml ice cold CM 1:1 v/v. After centrifugation the pellet was re-extracted twice with

0.5 ml CMW 10:10:3 v/v/v and the pooled extracts lyophilised. The dried glycolipids were redissolved in 1 ml water saturated n-butanol which was washed with 0.5 ml water. The butanol extract was then evaporated, and the glycolipids redissolved in CM 2:1 v/v and stored at −80 °C.

2.7. Analysis of UDP-[^{3}H]galactose-labelled glycoproteins and glycolipids in parasites

UDP-[^{3}H]galactose-labelled glycoproteins from labelled microsomal preparations were precipitated with ethanol, solubilised in Laemmli sample buffer and separated by SDS–PAGE. The gel was dried and fluorographed after impregnation with Enhance reagent (Perkin Elmer, USA). Glycolipids were separated by silica gel TLC using either CMW 65:25:4 v/v/v or 10:10:3 v/v/v, sprayed with Enhance and then fluorographed. Two microgram each of galactocerebroside, galactocerebroside sulphate (sulfoquinovosyldiacylglycerol), lactocerebroside, digalactosyl diglyceride and globotetraosylcerebroside (Sigma) dissolved in CM 2:1 v/v was used as migration markers for TLC.

3. Results

3.1. FITC-BSB4 lectin labelling of parasites

FITC-labelled BSB4 reacted with *P. falciparum* late asexual stages as reported previously (Ramasamy and Reese, 1986). Binding was observed to the merozoite surface (arrowed in Fig. 1, panel A) and to a compartment external to parasites undergoing schizogony within red cells (Fig. 1, panel A). Binding to *T. brucei* trypomastigotes occurred on the surface membrane and also internal compartments, possibly the endoplasmic reticulum and endosomes, but not the nucleus (Fig. 1, panel B). These results were suggestive of the presence of terminal α-gal containing glycoconjugates in both parasites.

3.2. Characterisation of parasite proteins binding to BSB4

Parasite proteins binding to BSB4-Sepharose beads were then characterised after elution from the beads with Laemmli sample buffer followed by SDS–PAGE, and probing with biotin-BSB4. Reactivity with potentially polydisperse material of M_r > 140 kDa and distinct species in the range of M_r 7–115 kDa, including a doublet of 58–60 kDa that could correspond to the VSG, was observed in *T. brucei* trypomastigote lysates (Fig. 2). In contrast, far fewer proteins at much lower quantities were bound by BSB4-Sepharose from unparasitized red cells and *P. falciparum* late stages compared to *T. brucei* trypomastigotes (Fig. 2). Some proteins appeared to react specifically, albeit weakly, in *P. falciparum* when compared with unparasitized red cells (arrowed in Fig. 2). Among these were proteins of similar M_r to known merozoite surface antigens and their breakdown products, including MSP1 (200, 125 kDa) and MSP2 (50, 55 kDa) (Gilson et al., 2006). BSB4 subunits with a calculated protein M_r of 28 kDa that migrate with an apparent M_r of 41 kDa due to glycosylation were also observed in the probed blots (Fig. 2, asterisk). Silver staining after SDS–PAGE also showed that far more proteins from *T. brucei* trypomastigotes than *P. falciparum* late blood stages or unparasitized red cells bound to BSB4-Sepharose (Supplementary Fig. S1).

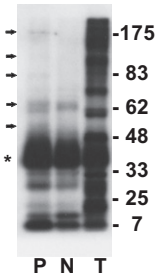

Fig. 2. Biotin-BSB4 lectin blot of parasite and control red cell proteins binding to BSB4-Sepharose. Proteins from 10^8 *P. falciparum* late stages or unparasitized red cells and 6×10^6 *T. brucei* trypomastigotes binding to BSB4-Sepharose beads were eluted with Laemmli buffer, separated by SDS–PAGE on 4–12% gradient gels and transferred to PVDF membrane. The blots were probed with biotin-BSB4 lectin, and developed with streptavidin-peroxidase and luminol and imaged on X-ray film. P – *P. falciparum* asexual blood late stages; N – unparasitized red cells and T – *T. brucei* bloodstream trypomastigotes. The asterisk indicates the migration position of eluted BSB4 lectin monomers of apparent M_r 41 kDa that also react with biotin-BSB4. The migration positions of molecular weight markers are shown.

The number of VSG molecules is estimated to be ≈10^7 and that of all other glycoproteins <10^6 per *T. brucei* trypomastigote (Ziegelbauer and Overath, 1993; Grünfelder et al., 2002). Based on the amount of MSP1 protein that can be isolated from parasitized red cells (Holder and Freeman, 1984), it can be estimated that MSP1 and MSP2, that constitute two thirds of the surface coat and half of all the GPI anchored proteins of merozoites (Gilson et al., 2006), are present at >10^5 molecules per schizont. Comparison between the level of BSB4 reactivity of proteins from 6×10^6 trypomastigotes and 10^8 malaria parasites (Fig. 2) suggested that at most only a minor fraction (estimated at <600 molecules per parasitized red cell) of MSP1 and MSP2 could be α-galactosylated to account for the level of BSB4-binding.

3.3. P. falciparum glycolipids binding to BSB4 and immune human antibodies

The weak or absence of binding of *P. falciparum* glycoproteins to BSB4 gave rise to the possibility that parasite glycolipids may be

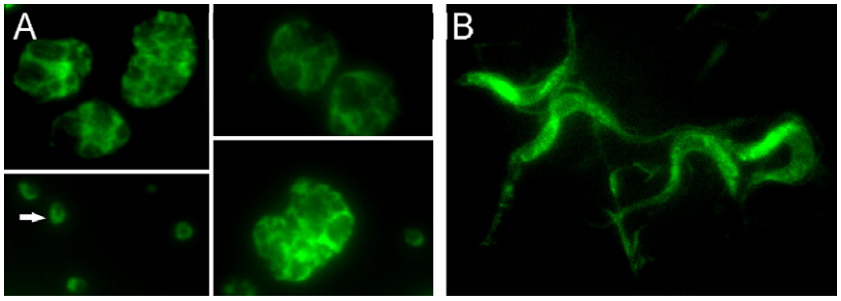

Fig. 1. Fluorescence photomicrograph of the reaction of FITC-BSB4 lectin with parasites. Parasites were stained with FITC-labelled BSB4 lectin specific for terminal α-galactosyl residues after isolation from culture and acetone/methanol fixation on microscope slides. Panels in A: *P. falciparum* late asexual stages grown in blood group O$^+$ red blood cells. The arrow shows the reaction of FITC-BSB4 with an extracellular merozoite. Panel B: *T. brucei* bloodstream form trypomastigotes. Viewed and photographed at 1000× magnification.

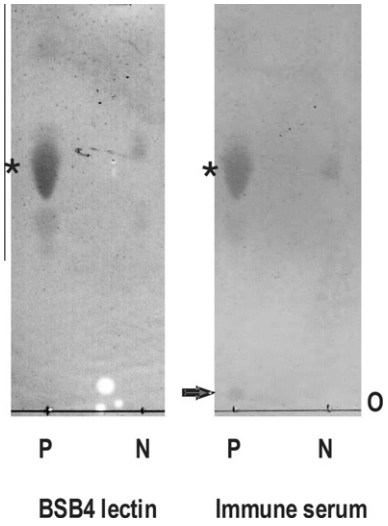

Fig. 3. Blot showing *P. falciparum* lipids reacting with BSB4 lectin and immune malaria serum. Glycolipids from 7.5×10^7 *P. falciparum* late asexual stages cultured in blood group O^+ erythrocytes [P], and control, uninfected blood group B^+ erythrocyte lipids [N] were separated on TLC using CMW 65:25:4 and reacted with biotinylated BSB4 lectin specific for terminal α-galactosyl residues, or pooled human sera from a malaria endemic area of Uganda. Bound Biotin-BSB4 was then reacted with streptavidin-peroxidase and the bound human antibodies with peroxidase-conjugated anti-human immunoglobulin before development with diaminobenzidine substrate. The asterisks denote the major reacting lipid species from parasites that also reacted with streptavidin-peroxidase alone or with non-immune human serum followed by treatment with peroxidase-conjugated anti-human immunoglobulin (Supplementary Fig. S2). The sample origin is indicated by O.

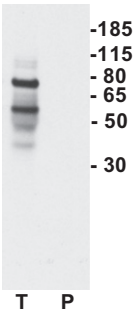

Fig. 4. Fluorograph of SDS–PAGE separation of parasite glycoproteins labelled in vitro in microsomes with UDP-[^{3}H]galactose. The lanes contained proteins derived from 10^6 *T. brucei* trypomastigotes (T) or *P. falciparum* late asexual blood stages (P). Fluorography was performed for 48 h. The migration positions of molecular weight markers in kDa are shown on the right.

responsible for the binding of FITC-BSB4 to parasites. This and the possibility that the glycolipids might be immunogenic in humans were investigated next. Glycolipids isolated from *P. falciparum* late asexual stages and unparasitized red cells were separated by thin layer chromatography (TLC) and reacted with biotin-labelled BSB4 and immune and control non-immune human sera in overlays. A major *P. falciparum* lipidic component (R_f of 0.34 in CMW 65:25:4 v/v/v) reacted with BSB4 and immune human sera (asterisk in Fig. 3). However this material also reacted with streptavidin-peroxidase alone and with non-immune human sera indicating that the reactivity with BSB4 and immune human sera was not due to the presence of α-gal or specific antigenicity (Supplementary Fig. S2). This species is apparently present in significantly lower quantity in an equivalent number of unparasitized red cells, and migrated with mobility between that of globotetraosylcerebroside and galactocerebroside sulphate in CMW 65:25:4 v/v/v and did not react with orcinol reagent. This suggests that it is not a glycolipid. Other lipids in *P. falciparum* also appeared to bind biotin-BSB4 but there was corresponding, albeit weaker, binding to similarly migrating lipids in unparasitized red cells (Fig. 3). Therefore no parasite-specific glycolipid that reacted with BSB4-lectin could be clearly discerned in these overlay assays.

However a hydrophilic lipid moiety with R_f of approximately 0.05 in CMW 65:25:4 v/v/v and specific to *P. falciparum*, reacted weakly with immune human sera (arrowed in Fig. 3) but not with BSB4 (Fig. 3) or non-immune human sera (Supplementary Fig. S2).

3.4. Glycoproteins labelled with UDP-[^{3}H]galactose in microsomal preparations

The labelling of *T. brucei* and *P. falciparum* glycoproteins and glycolipids with UDP-[^{3}H]galactose in vitro using microsomal preparations was investigated in a different approach to identify molecules containing α-gal in the parasites. While the incorporation of [^{3}H]galactose into *T. brucei* glycoproteins was strong, this was patently not the case for *P. falciparum* glycoproteins (Fig. 4) indicating that, consistent with the low or no BSB4-binding, terminally-linked galactose was not detectable in protein-linked oligosaccharides of *P. falciparum*. The estimated molecular weights of the two strongly labelled *T. brucei* glycoproteins in microsomal preparations were 75 and 56 kDa. Other proteins of 105 (doublet), 42 and 35 kDa and polydisperse material in the M_r range 50–65 kDa were also labelled. On a longer exposure of fluorographs of non-ethanol precipitated labelled microsomes, additional proteins were seen to be labelled with UDP-[^{3}H]galactose in *T. brucei* but only material migrating near the SDS–PAGE dye front in *P. falciparum* that was also present in unparasitized red cells (Supplementary Fig. S3). It can be estimated that specific galactosylation of *P. falciparum* proteins does not occur to the level of galactosylation of the weakest in vitro labelled *T. brucei* glycoproteins that are expected to be present at much less than 10^5 copies per cell (Ziegelbauer and Overath, 1993; Grünfelder et al., 2002).

The presence of an inhibitory factor in the *P. falciparum* membranes that prevents the transfer of [^{3}H]galactose to macromolecular acceptors could be excluded as *T. brucei* glycoproteins labelled well when *P. falciparum* and *T. brucei* microsomes were mixed together (Supplementary Fig. S3). Partial removal of radioactivity from [^{3}H]galactose-labelled *T. brucei* glycoproteins by limited digestion of labelled microsomes with coffee bean α-galactosidase was consistent with the presence of α-gal (Supplementary Fig. S4).

3.5. Labelling of glycolipids with UDP-[^{3}H]galactose in microsomal preparations

P. falciparum late asexual blood stages and unparasitized red cells incorporated [^{3}H]galactose into an identically migrating glycolipid with an average R_f of 0.22 in CMW 65:25:4 v/v/v (Fig. 5). This glycolipid migrated as a doublet in other TLC separations. More hydrophobic glycolipids were also weakly labelled with similar intensities in both *P. falciparum* and control red cells. In contrast, *T. brucei* bloodstream trypomastigotes showed two prominently labelled glycolipids of R_f 0.21 and 0.47 as well as a more hydrophobic glycolipid of R_f 0.83 in CMW 65:25:4 v/v/v that migrated with the galactocerebroside marker. Radioactivity in the more hydrophilic *T. brucei* glycolipid was sensitive to α-galactosidase (Supplementary Fig. S5) as well as nitrous acid treatment and saponification (Supplementary Fig. S6) consistent with a galactosylated GIPL structure reported previously (Mayor et al., 1992; Guther et al., 2003).

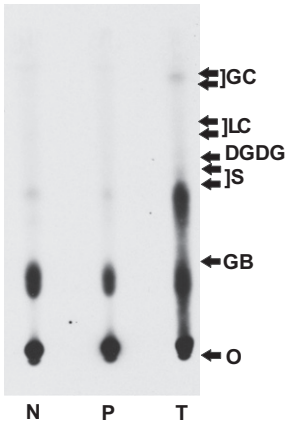

Fig. 5. Fluorograph of UDP-[^{3}H]galactose labelled glycolipids. Labelled glycolipids were separated by silica gel TLC using CMW 65:25:4 as solvent. Each lane contained 33 kBq of radioactivity. P – *P. falciparum* asexual blood late stages; N – unparasitised red cells used to culture P, and T – *T. brucei* bloodstream trypomastigotes. The migration positions of glycolipid standards used in the TLC are shown by arrows. GC – galactocerebroside (doublet); LC – lactocerebroside (doublet); DGDG – digalactosyl diglyceride; S – sulfoquinovosyldiacylglycerol (doublet); GB – globotetraosylcerebroside; O – sample origin.

3.6. Genome search for an α-galactosyl transferase in P. falciparum

In another approach, we attempted to identify a α-galactosyl transferase in the *P. falciparum* genome (GeneDB, Wellcome-Sanger Institute) by protein BLAST searches with different α-galactosyl transferases. Murine α-1, 3-galactosyl transferases (NP_001139293 and NP_034413.2) as well as murine cis-A, B transferase (BAB20560.1) did not yield homologues in *P. falciparum* and other malaria parasites. A BLAST search with DGDG synthase from *Arabidopsis thaliana* (NP_191964) detected weak homology ($E = 0.28$) with PF10_0316 which is annotated as an N-acetylglucosaminyl phosphatidyl inositol synthase or PIG A, an enzyme functioning in the first step of the synthesis of the precursor to GPI anchors. A BLAST search with *Staphylococcus aureus* galactosyl transferase (YP_039597.1) however detected PF10_0316 as being very significantly homologous ($E = 10^{-10}$). The *P. falciparum* genome therefore appears to lack a discernable α-galactosyl transferase gene.

4. Discussion

BSB4 is a lectin that shows specificity for terminal α-galactopyranosyl residues, particularly in α1–3 and α1–4 linkages (Kirkeby and Moe, 2001). The binding of FITC-BSB4 to *P. falciparum* mature asexual blood stages and merozoites therefore initially suggested the presence of α-gal-containing glycoconjugates in the parasites, consistent with some previous observations (Ramasamy and Reese, 1985, 1986; Jakobsen et al., 1987; Ravindran et al., 1988; Ramasamy and Rajakaruna, 1997; Mya et al., 2002). However the much lower quantities of *P. falciparum* proteins that bound to BSB4-Sepharose compared to *T. brucei* glycoproteins, indicated that there were either relatively few molecules of α-gal containing glycoproteins in the malaria parasite or that the observed weak binding was due to interactions that are not dependent on the presence of α-gal.

UDP-galactose is the normal activated precursor of galactose required for incorporation into glycoproteins and glycolipids in eukaryotic cells. Under the experimental conditions utilised, the galactose from UDP-galactose would almost exclusively be added to the non-reducing termini of oligosaccharide chains of glycoproteins and glycolipids. The transfer to acceptor molecules typically occurs in membranes of the Golgi body through the action of a membrane bound galactosyl transferase. However galactose was not detectably incorporated into proteins when UDP-galactose was used as a source in microsomes prepared from *P. falciparum*, under conditions where *T. brucei* bloodstream trypomastigotes efficiently incorporate galactose into glycoproteins. *P. falciparum* microsomes also did not contain components that inhibit the incorporation of galactose from UDP-galactose into *T. brucei* trypomastigote glycoproteins. The results therefore suggest that if terminal α-galactosylation does occur in the GPI anchor or oligosaccharide side chains of *P. falciparum* late asexual blood stage proteins, the rate and levels of incorporation are lower than in any of the galactosylated *T. brucei* glycoproteins detected in this study.

The presence of glycolipids bearing α-gal in late asexual blood stages of *P. falciparum* was investigated by BSB4 lectin overlay assays after TLC separation of unlabelled glycolipids from the parasite, and by examining the incorporation of [^{3}H]galactose from UDP-[^{3}H]galactose into glycolipids in microsomes in vitro. The results showed no evidence for the specific synthesis of galactosylated glycolipids by parasite microsomes. Rather, [^{3}H]galactose was incorporated into glycolipids of similar R_f in microsomes from malaria parasites and unparasitized red cells, suggesting that the labelled acceptors were lipid components of the host red cells. The β-D-galactosyl transferases present in red cell membranes (Cartron et al., 1978) are likely to be responsible for transferring [^{3}H]galactose from UDP-[^{3}H]galactose to acceptor red cell membrane glycolipids in the microsomes from both parasitized and unparasitized red cells. The in vitro labelling experiments did not detect the synthesis of DGDG, MGDG and monogalactosyl cerebroside reported previously in microsomes from *P. falciparum* late asexual blood stages (Marechal et al., 2002). In a follow-up study, a DGDG-like epitope was reported to be expressed on merozoite membranes (Botte et al., 2008). However the present study did not identify a *P. falciparum* α-galactosyl transferase homologous to a plant α-galactosyl transferase responsible for DGDG synthesis in a BLAST search. The earlier study by Marechal et al., 2002 did not detect the common hydrophilic galactosylated lipid in parasitized and unparasitized red cell that was detected by labelling microsomes with UDP-[^{3}H]galactose in the present work. A different parasite isolate was used and detection of radiolabeled glycolipids was performed with an automated TLC scanner and their characterisation based on TLC migration properties and reactivity with anti-DGDG antibody, in the other study (Marechal et al., 2002). These and other methodological differences may be responsible for this discrepancy, which merits further investigation.

There is an indication from lectin blots of lipids separated by TLC that human antibodies from endemic-area sera react with a relatively hydrophilic lipid present in parasitized and not unparasitized red cells. It is possible that this is a GIPL that is structurally related to or serves as a precursor for the GPI anchor of *P. falciparum* glycoproteins which is known to elicit antibodies in humans (Gerold et al., 1994; Gowda et al., 1997). Free GIPLs have been characterised in many parasitic protozoa (Mayor et al., 1992; McConville and Ferguson, 1993; Ferguson, 1999; Guther et al., 2003; Mendonc-Previato et al., 2009) and their possible presence also in *P. falciparum* merits further investigation since they may, like GPI anchors, have a role in malaria immunity and pathogenesis.

An uncharacterised lipid that shows greater non-specific reactivity in TLC plates with several proteins, including BSB4, in parasites compared to unparasitized red cells was also observed. Proteins can interact non-specifically with high concentrations of lipids through conformational changes exposing normally internal hydrophobic residues. It is possible therefore that this lipid present in high concentrations in asexual blood stages of *P. falciparum* is at least partly responsible for the non-specific binding of FITC-BSB4 in aqueous solution to parasites. This non-specific interaction might

be expected to be minimal when 0.5% Triton X-100 solutions of parasites are used for the BSB4-Sepharose binding assay.

Bioinformatics analysis of *P. falciparum* genome sequences suggests that it possesses genes coding for all of the enzymes involved in GPI anchor synthesis (Delorenzi et al., 2002) but only the genes responsible for the synthesis of dolichol-PP-GlcNAc and oligosaccharyl transferase in the N-linked glycosylation pathway (Samuelson et al., 2005). These results are consistent with the lack of extensive N- or O-linked glycosylation in *P. falciparum* other than the incorporation of GlcNAc as concluded in recent reviews (von Itzstein et al., 2008; de Macedo et al., 2010). No candidate *P. falciparum* genes for the synthesis of α-gal were identified through bioinformatics searches, in agreement with the laboratory findings. The homology of *P. falciparum* N-acetylglucosaminyl phosphatidyl inositol synthase with *S. aureus* galactosyl transferase on the other hand likely reflects its evolutionary origin.

Our data therefore provide no evidence for a detectable presence of terminal α-gal containing glycoconjugates that are specific to *P. falciparum,* and suggest that some earlier observations to the contrary may have been due to non-specific binding of BSB4 lectin in aqueous solution to parasite lipids. Based on the sensitivity of the methods employed for detection, we suggest that terminal α-gal containing glycoproteins cannot be present at >600 molecules per late stage *P. falciparum* cell, and that α-gal containing *P. falciparum* glycolipids, if present, are synthesised at lower rates in vitro than the putative α-gal containing GIPL or the more weakly labelled hydrophobic glycolipid migrating with galactocerebroside of *T. brucei* .

In contrast, there was clear evidence for terminal galactosylated glycoproteins and glycolipids in *T. brucei.* Two different α-galactosyl transferase activities are detectable in *T. brucei* (Pingel et al., 1995, 1999), but there is no report to date on their gene sequences. The preferred acceptor for one of the α-galactosyl transferases was N-acetyl lactosamine (Pingel et al., 1999) while for the other it was an α-mannoside which resembles the acceptor on the GPI anchor (Pingel et al., 1995). The major glycoproteins that strongly incorporated [^{3}H]galactose in the present study included those with M_r compatible with the ≈50 kDa M_r VSG (McConville and Ferguson, 1993) and a trans-membrane anchored 75 kDa invariant surface glycoprotein (Ziegelbauer and Overath, 1993) that are known to be present on the surface of the trypomastigotes. The class 2 VSG of *T. brucei* strain 221 of approximate M_r 58 kDa used in the present study contains α1–2 and α1–6 linked terminal galactosyl residues in the GPI anchor (Mehlert et al., 1988), consistent with this labelling pattern and also the relatively weaker binding to BSB4 as seen in the lectin blot (Fig. 2). The additional galactosylated proteins in *T. brucei* are also likely to be at least partly expressed on the plasma membrane and therefore may be important from an immunological perspective. Many trypomastigote glycoproteins contain N-linked polylactosamine chains that, like the VSG (Zamze et al., 1991), may be partly capped by α-gal although it has been argued that extensive capping with α1–3 gal is not possible because of recognition by natural anti- α1–3 gal bodies in human serum (Atrih et al., 2005). Some α-galactosylation can also occur on the GPI anchors of non-VSG *T. brucei* proteins as demonstrated for the transferrin receptor (Mehlert and Ferguson, 2007). It is also conceivable that a degree of unphysiological galactosylation of glycoconjugates may occur in microsomes due to the disruption of normal membrane structures. However the binding of many trypomastigote proteins to BSB4 in the fluorescence, affinity bead and blotting assays suggests that much of the observed galactosylation occurs naturally. The properties of the two more hydrophilic glycolipid components in trypomastigotes with R_f of 0.21 and 0.47 in CMW 65:25:4 v/v/v are consistent with them being the galactosylated versions of GIPLs related to GPI anchors detected previously, with the more hydrophobic glycolipid being the inositol-acylated version of the other (Mayor et al., 1992; Guther et al., 2003). The most hydrophobic glycolipid labelled with [^{3}H]galactose in the trypomastigotes migrated with the same R_f as galactocerebroside. Although the synthesis of glucocerebroside in *T. brucei* trypomastigotes was reported previously (Uemura et al., 2006), it is possible that galactocerbroside is also synthesised by the parasites. Characterisation by mass spectrometry will be required to further establish the structures of the galactosylated glycolipids of *T. brucei* detected in the present study.

Acknowledgments

We are grateful to M. Grainger and Drs A.A. Holder, S. Campino and T. Tiffert for generously providing parasite material, to Prof D. Dunne for the human sera and to members of the Field lab for other support. This work was supported by a Royal Society short-term visiting scientist grant and a research grant from the Universiti Brunei Darussalam to RR and benefited from Wellcome Trust project and program grant support to MCF.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.exppara.2012.02.017.

References

Almeida, I.C., Ferguson, M.A.J., Shenkman, S., Travassos, L.R., 1994. Lytic anti-α-galactosyl antibodies from patients with chronic Chagas' disease recognise novel O-linked oligosaccharides on mucin-like glycosyl-phosphatidylinositol-anchored glycoproteins of *Trypanosoma brucei*. Biochemical Journal 304, 793–802.

Atrih, A., Richardson, J.M., Prescott, A.R., Ferguson, M.A.J., 2005. *Trypanosoma brucei* glycoproteins contain novel giant poly-N-acetyllactosamine carbohydrate chains. Journal of Biological Chemistry 280 (2), 865–871.

Botte, C., Saidani, N., Mondragon, R., Mondragon, M., Isaac, G., Mui, E., McLeod, R., Dubremetz, J.F., Vial, H., Welti, R., Cesbron-Delauw, M.F., Mercier, C., Marechal, E., 2008. Subcellular localisation and dynamics of a digalactoside-like epitope in *Toxoplasma gondii*. Journal of Lipid Research 49, 746–762.

Buscaglia, C.A., Campo, V.A., Frasch, A.C.C., Di Noia, J.M., 2006. Trypanosoma cruzi surface mucins: host-dependent coat diversity. Nature Reviews Microbiology 4, 229–236.

Cartron, J.P., Cartron, J., Andreu, G., Salmon, Ch., Bird, G.W.G., 1978. Selective deficiency of 3-β-D-galactosyltransferase (T-transferase) in Tn-polyagglutinable erythrocytes. The Lancet, 856–857.

Christie, W.W., 1993. Preparation of lipid extracts from tissues. In: Christie, W.W. (Ed.), Advances in Lipid Methodology, vol. 2. Oily Press, Dundee, pp. 195–213.

Chung, W.L., Leung, K.F., Carrington, M., Field, M.C., 2008. Position-specific ubiquitylation is required for internalisation and degradation of trans-membrane surface proteins in trypanosomes. Traffic 9, 1681–1697.

Couto, A.S., Caffaro, C., Uhrig, M.L., Kimura, E., Peres, V.J., Merino, E.F., 2004. Glycosphingolipids in *Plasmodium falciparum*. Presence of an active glucosylceramide synthase. European Journal of Biochemistry 271, 2204–2214.

de Macedo, C.S., Schwarz, R.T., Todeschini, A.R., Previato, J.O., Mendonça-Previato, L., 2010. Overlooked post-translational modifications of proteins in Plasmodium falciparum: N- and O-glycosylation – a review. Memórias do Instituto Oswaldo Cruz, Rio de Janeiro. 105 (8), 949–956.

Delorenzi, M., Sexton, A., Shams-Eldin, H., Schwarz, R.T., Speed, T., Schofield, L., 2002. Genes for glycosylphosphatidylinositol toxin biosynthesis in *Plasmodium falciparum*. Infection and Immunity 70 (8), 4510–4522.

Ferguson, M.A.J., Homans, S.W., Dwek, R.A., Rademacher, T.W., 1988. Glycosyl-phosphatidylinositol moiety that anchors *Trypanosoma brucei* variant surface glycoprotein to the membrane. Science 239, 753–759.

Ferguson, M.A.J., 1999. The structure, biosynthesis and functions of glycosylphosphatidylinositols anchors, and the contributions of trypanosome research. Journal of Cell Science 112, 2799–2809.

Foth, B.J., McFadden, G.I., 2003. The apicoplasts: a plastid in *Plasmodium falciparum* and other apicomplexan parasites. International Review of Cytology 224, 57–110.

Gerold, P., Dieckmann-Schuppert, A., Schwarz, R.T., 1994. Glycosylphosphatidylinositols synthesized by asexual erythrocytic stages of the malaria parasite, *Plasmodium falciparum*. Candidate for plasmodial glycosylphosphatidylinositols membrane anchor precursors and pathogenicity factors. Journal of Biological Chemistry 269, 2597–2606.

Gerold, P., Schwartz, R.T., 2001. Biosynthesis of glycosphingolipids de-novo by the human malaria parasite *Plasmodium falciparum*. Molecular and Biochemical Parasitology 112, 29–37.

Gilson, P.R., Nebl, T., Vukcevic, D., Moritz, R.L., Sargeant, T., Speed, T.P., Schofield, L., Crabb, B.S., 2006. Identification and stoichiometry of glycosylphosphatidylinositols anchored membrane proteins of the human malaria parasite *Plasmodium falciparum*. Molecular and Cellular Proteomics 5, 1286–1299.

Gowda, D.C., Gupta, P., Davidson, E.A., 1997. Glycosylphosphatidylinositols represent the major carbohydrate modification in protein of intraerythrocytic stage *Plasmodium falciparum*. Journal of Biological Chemistry 272, 6428–6439.

Grünfelder, C.G., Engstler, M., Weise, F., Schwarz, H., Stierhof, Y.D., Boshart, M., Overath, P., 2002. Accumulation of a GPI-anchored protein at the cell surface requires sorting at multiple intracellular levels. Traffic 3 (8), 547–559.

Guther, M.L., Prescott, A.R., Ferguson, M.A., 2003. Deletion of the GPIdeAc gene alters the location and fate of glycosylphosphatidylinositol precursors in *Trypanosoma brucei*. Biochemistry 42 (49), 14532–14540.

Hansen, D.S., Siomos, M.A., Buckingham, L., Scalzo, A.A., Schofield, L., 2003. Regulation of murine cerebral malaria pathogenesis by CD-1d restricted NKT cells and the natural killer complex. Immunity 18, 391–402.

Harwood, J.L., 1980. Plant acyl lipids. In: Stumpf, P.K. (Ed.), The Biochemistry of Plants, vol. 4. Academic Press, New York, pp. 1–55.

Holder, A.A., Freeman, R.R., 1984. The three major antigens on the surface of *Plasmodium falciparum* merozoites are derived from a single high molecular weight precursor. Journal of Experimental Medicine 160, 624–629.

Jakobsen, P.H., Theander, T.G., Jensen, J.B., Molbek, K., Jepsen, S., 1987. Soluble *Plasmodium falciparum* antigens contain carbohydrate moieties important for human reactivity. Journal of Clinical Microbiology 25 (11), 2075–2079.

Kirkeby, S., Moe, D., 2001. Binding of *Griffonia simplicifolia* 1 isolectin B4 (GS1 B4) to α-galactose antigens. Immunology and Cell Biology 79, 121–127.

Krishnegowda, G., Hajjar, A.M., Zhu, J., Douglass, E.J., Uematsu, S., Akira, S., Woods, A.S., Gowda, D.C., 2005. Induction of proinflammatory responses in macrophages by the glycosylphosphatidylinositols of *Plasmodium falciparum*: cell signalling receptors, glycosylphosphatidylinositol (GPI) structural requirement, and regulation of GPI activity. Journal of Biological Chemistry 280, 8606–8616.

Landoni, M., Duschak, V.G., Peres, V.J., Nonamid, H., Erra-Balsells, R., Katzin, A.M., Couto, A.S., 2007. *Plasmodium falciparum* biosynthesizes sulfoglycosphingolipids. Molecular and Biochemical Parasitology 154, 22–29.

Marechal, E., Azzouz, N., de Macedo, C.S., Block, M.A., Feagin, J.E., Schwartz, R.T., Joyard, J., 2002. Synthesis of chloroplast galactolipids in apicomplexan parasites. Eukaryotic Cell 1 (4), 653–656.

Masterson, W.J., Raper, J., Doerling, T.L., Hart, G.W., Englund, P.T., 1990. Fatty acid remodelling: a novel reaction sequence in the biosynthesis of trypanosome glycosyl phosphatidylinositol membrane anchors. Cell 62, 73–80.

Mayor, S., Menon, A.K., Cross, G.A.M., 1992. Galactose-containing glycosylphosphatidylinositols in *Trypanosoma brucei*. Journal of Biological Chemistry 267, 754–761.

McConville, M.J., Ferguson, M.A.J., 1993. The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. Biochemical Journal 294, 305–324.

Mehlert, A., Richardson, J.M., Ferguson, M.A.J., 1988. Structure of the glycophosphatidyl inositol membrane anchor of a class-2 variant surface glycoprotein of *Trypanosoma brucei*. Journal of Molecular Biology 277, 370–392.

Mehlert, A., Ferguson, M.A.J., 2007. Structure of the glycosylphosphatidylinositol anchor of the *Trypanosoma brucei* transferrin receptor. Molecular and Biochemical Parasitology 151 (2), 220–223.

Mendonc-Previato, L., Todeschini, A.R., Heise, N., Previato, J.O., 2009. Protozoan parasite-specific carbohydrate structures. Current Opinion in Structural Biology 15, 499–505.

Mya, M.M., Roy, A., Saxena, R.K., Roy, K.B., 2002. Isolation, part characterisation, immunogenicity and specificity study of *Plasmodium falciparum* culture supernatant. Japan Journal of Infectious Diseases 55, 150–156.

Naik, R.S., Branch, O.L.H., Woods, A.S., Vijaykumar, M., Perkins, D.J., Lal, A.A., Cotter, R.J., Costello, C.E., Ockenhouse, C.F., Davidson, E.A., Gowda, D.C., 2000. Glycophosphatidyl anchors of *Plasmodium falciparum*: molecular characterisation and naturally elicited antibody response that may provide immunity to malaria pathogenesis. Journal of Experimental Medicine 192 (11), 1563–1575.

Naus, C.W.A., Jones, F.M., Satti, M.Z., Joseph, S., Riley, E.M., Kimani, G., Mwatha, J.K., Kariuki, C.H., Ouma, J.H., Kabatereine, N.B., Vennervald, B.J., Dunne, D.W., 2003. Serological responses among individuals in areas where both schistosomaisis and malaria are endemic: cross-reactivity between *Schistosoma mansoni* and *Plasmodium falciparum*. Journal of Infectious Diseases 187, 1272–1282.

Pingel, S., Field, R.A., Guther, M.L.S., Duszenko, M., Ferguson, M.A.J., 1995. The hydrophobic mannoside Man αl–6 Man α-S-(CH2)7-CH3 acts as an acceptor for the UDP-Gal:glycosylphosphatidylinositol anchor alpha 1,3-galactosyltransferase of *Trypanosoma brucei*. Biochemical Journal 309, 877–882.

Pingel, S., Rheinweiler, U., Kolb, V., Duszenko, M., 1999. Purification and characterisation of an α-galactosyl transferase from *Trypanosoma brucei*. Biochemical Journal 338, 545–551.

Ramasamy, R., Rajakaruna, R., 1997. Association of malaria with 1–3 galactosyl transferase inactivation in catarrhines. Biochimica et Biophysica Acta 1360, 241–246.

Ramasamy, R., Reese, R.T., 1985. A role for carobohydrate moieties in the immune response to malaria. Journal of Immunology 134, 1952–1955.

Ramasamy, R., Reese, R.T., 1986. Terminal galactose residues and the antigenicity of *Plasmodium falciparum* glycoproteins. Molecular and Biochemical Parasitology 19, 91–101.

Ravindran, B., Satapathy, A.K., Das, M.K., 1988. Naturally-occurring anti-α-galactosyl antibodies in human *Plasmodium falciparum* infections – a possible role for autoantibodies in malaria. Immunology Letters 19, 137–142.

Samuelson, J., Banerjee, S., Magnell, P., Cui, J., Kelleher, J., Gilmore, R., Robbins, P.W., 2005. The diversity of dolichol-linked precursors to Asn-linked glycans likely results from secondary loss of sets of glycosyltransferases. Proceedings of the National Academy of Sciences USA 102 (5), 1548–1553.

Schofield, L., Hewitt, M.C., Evans, K., Slomos, M.A., Seeburgher, P.H., 2002. Synthetic GPI as candidate anti-toxic vaccine in model of malaria. Nature 418, 785–789.

Schofield, L., McConville, M.J., Hansen, D., Campbell, A.S., Fraser-Reid, B., Grusby, M.J., Tachado, S.D., 1999. CD1d-restricted immunoglobulin G formation to GPI-anchored antigens mediated by NKT cells. Science 283, 225–228.

Tachado, S.D., Gerold, P., McConville, M.J., Baldwin, T., Quilici, D., Schwarz, R.T., Schofield, L., 1996. Glycosylphosphatidylinositol toxin of *Plasmodium* induces nitric oxide synthase expression in macrophages and vascular endothelial cells by a protein tyrosine kinase-dependent and protein kinase C-dependent signalling pathway. Journal of Immunology 156, 1897–1907.

Trager, W., Jensen, J.B., 1976. Human malaria parasites in continuous culture. Science 193, 673–675.

Tupin, E., Kinjo, Y., Kronenberg, M., 2007. The unique role of natural killer T cells in the immune response to microorganisms. Nature Reviews Microbiology 5, 405–417.

Uemura, A., Watarai, S., Kushi, Y., Kasama, T., Ohnishi, Y., Kodama, H., 2006. Analysis of neutral glycosphingolipids from *Trypanosoma brucei*. Veterinary Parasitology 140 (3–4), 264–272.

Vivas, L., O' Dea, K.P., Noya, O., Pabon, R., Magres, M., Botto, C., Holder, A.A., Brown, K.N., 2008. Hyper reactive malarial splenomegaly is associated with low levels of antibodies against red blood cell and *Plasmodium falciparum* derived glycolipids in Yanomami Amerindians from Venezuela. Acta Tropica 105, 207–214.

von Itzstein, M., Plebanski, M., Cooke, B.M., Coppel, R.L., 2008. Hot, sticky and sweet: the glycobiology of *Plasmodium falciparum*. Trends in Parasitology 24 (5), 211–218.

World Health Organisation 2010a. Fact sheet No. 94 Malaria. Genève.

World Health Organisation 2010b. Fact sheet No 259 African trypanosomiasis (sleeping sickness). Genève.

Zamze, S.E., Ashford, D.A., Wooten, E.W., Rademacher, T.W., Dwek, R.A., 1991. Structural characterization of the asparagine-linked oligosaccharides from *Trypanosoma brucei* type II and type III variant surface glycoproteins. Journal of Biological Chemistry 266 (30), 20244–20261.

Ziegelbauer, K., Overath, P., 1993. Organization of two invariant surface glycoproteins in the surface coat of *Trypanosoma brucei*. Infection and Immunity 61 (11), 4540–4545.