HUMAN SERUM HAPTOGLOBIN IS TOXIC TO *PLASMODIUM FALCIPARUM IN VITRO*

HEATHER IMRIE*, DAVID. J. P. FERGUSON[+] AND KAREN P DAY*

**Peter Medawar Building for Pathogen Research and Department of Zoology, University of Oxford, OX1 3SY, Oxford, UK.*

[+]Department of Pathology and Bacteriology, John Radcliffe Hospital, OX39DU, Oxford, UK

Correspondence should be addressed to Heather Imrie;

Tel: +44 1865 271247

Fax: +44 1865 281898

Email: heather.imrie@medawar.ox.ac.uk

Abbreviations

TNF; Tumour necrosis factor

Abstract

Innate immune responses are important in the control of malaria, particularly in those who have not yet mounted an effective adaptive response. Here we report that the human serum acute phase protein, haptoglobin is toxic to *Plasmodium falciparum* cultured *in vitro*. This effect is phenotype-dependent and occurs during the trophozoite phase of the asexual life cycle. We propose that the increased levels of haptoglobin seen in the acute phase response may be protective against malaria in humans.

Keywords

Haptoglobin; *Plasmodium falciparum*; acute phase proteins

1. Introduction

Haptoglobin is an acute phase protein which is normally present in plasma at a concentration of 0.3–2 mg mL^{-1}, although levels may increase to 8 mg mL^{-1} during the acute phase response (reviewed [1]). It occurs in three phenotypes, the globular Hp1-1, the linear polymer Hp2-1 and the circular polymer Hp2-2, only one form being found in an individual [2]. Haptoglobin binds specifically and irreversibly to free haemoglobin and prevents haemoglobin-initiated oxidative damage in renal tissue [3], Hp2-2 and Hp2-1 binding less haemoglobin than Hp1-1 [4]. In recent years haptoglobin has been found to have various other functions, e.g. inhibition of prostaglandin synthesis [5], promotion of angiogenesis [6], antibody-like agglutination of *Streptococcus pyogenes* strains carrying the membrane antigen, T4 [7], inhibition of lectin-induced lymphocyte transformation, mediated by binding to

CD22 [8], promoting Th1-dominant T cell responses, possibly mediated via CD11b [9] and inhibition of the respiratory burst by phagocytes, mediated via CD11b [10]. More recently Hunt *et al.* have demonstrated a possible role for haptoglobin in control of malaria *in vivo* in mouse models [11] and thus we have investigated the possibility that haptoglobin may have a direct toxic effect on *P. falciparum*.

2. Materials and Methods

2.1. Parasite Cultures

P. falciparum isolates were obtained in Papua New Guinea and cultured *in vitro* according to the method of Trager and Jensen [12] with modifications by Day *et al.* [13], viz 5% haematocrit in buffered RPMI medium supplemented with 5% pooled human serum (previously screened for its ability to support parasite growth). The parasites were synchronised by 'plasma gel' selection [14], followed by sorbitol lysis in the next cycle [15] and cultures prepared in 50µl aliquots with 1% parasitised erythrocytes.

2.2. Flow Cytometry

When the parasites were at the late ring stage of development (18-24 hours after merozoite invasion of erythrocytes), various concentrations of haptoglobin (both pooled and phenotype-specific, obtained from Sigma Chemical Co., Poole, UK) were added to the cultures (0–5 mg mL^{-1} final concentration). After 24 hours, when the parasites were schizonts, the haptoglobin-enriched medium was removed and replaced with fresh medium. The parasites were allowed to go through schizogony and to invade erythrocytes. Parasite replication was assessed 60 hours after the

addition of haptoglobin; parasitised cells were fluorescence-labeled with ethidium bromide, cultures were then fixed with 0.5% paraformaldehyde and parasitaemias determined using the Coulter, Epics XL flow cytometer after the method of Piper *et al.* [16]. In a separate experiment, parasites were synchronized and divided into subcultures. Within each subculture it was estimated that parasites ranged in time-post-invasion by about 6 hours, as judged by light microscopy. Haptoglobin, at various concentrations was added, for 8-hour periods only, to different subcultures (0-8 hours, 8-16 hours, 16-24 hours, 24-32 hours, 32-40 hours and 40-48 hours post sorbitol treatment). The parasites completed one re-invasion cycle and were then counted.

2.3. Tritiated Hypoxanthine Uptake

Haptoglobin was added to cultures as above, but in addition tritiated hypoxanthine (Amersham, Little Chalfont, UK) was added (3μ Curie mL^{-1}). Scintillation counts were determined following cell harvesting at various time points after the addition of haptoglobin. Thus it was possible to compare levels of nucleic acid production in cultures.

2.4. Light and electron microscopy of infected erythrocytes

Synchronous cultures of *P. falciparum* infected erythrocytes at the late ring stage (18-24 hours post invasion) were divided into aliquots and cultured in media with or without 5mg/ml of haptoglobin for 24 hours. Thin smears were prepared, fixed with methanol and stained with Giemsa. Parasites were also examined by electron microscopy; samples were fixed in 2.5% gluteraldehyde in 0.1M cacodylate buffer

and post-fixed in 1% osmium tetroxide, dehydrated in ethanol, treated with propylene oxide and embedded in Spurr's epoxy resin. Thin sections were stained with uranyl acetate and lead citrate prior to examination in the JEOL 1200EX transmission electron microscope.

2.5. Biotinylation of haptoglobin

A solution of sulpho-NHS-biotin (Pierce) was prepared in PBS (10mM) immediately before use and added to pooled haptoglobin (5 mg mL^{-1}; Sigma) or to the control proteins: human albumin (5 mg mL^{-1}; Sigma) and anti-glycophorin A (0.05 mg mL^{-1}; Becton Dickinson). Reaction mixes were placed on ice for 2 hours, then dialysed to remove unbound biotin and concentrated using Microcon YM-50 centrifugal filter devises (Millipore Corporation). Parasite cultures were treated with plasma gel to obtain erythrocytes with 15% parasitaemia of mixed stages from late rings to mid trophozoites. Subcultures were incubated with labelled haptoglobin or albumin (5 mg mL^{-1}) or anti-glycophorin A (0.05 mg/ml) for 2 hours at 4°C and 37°c or for 8 hours at 37°C. Following incubation erythrocytes were washed in human tonicity PBS and re-suspended in PBS with protease inhibitors (Roche). Some cultures were treated with 0.05% saponin (ICN Biochemicals) and the erythrocyte lysate and washed parasite pellets obtained. Whole parasitised erythrocytes, erythrocyte lysates and parasite pellets were subjected to SDS polyacrylamide gel electrophoresis under reducing conditions. Following blotting onto nitrocellulose membranes, the biotin conjugates were detected by streptavidin-HRP (200 ng mL^{-1}; Pierce) and ECL (Amersham).

2.6. Direct and indirect fluorescence microscopy

Direct labelling of pooled haptoglobin and Haptoglobin 1-1 with fluorescein isothiocyanate (FITC) was performed using the FluoroTag^{Tm} FITC conjugation kit (Sigma). The molar FITC: protein ratio for Hp1-1 was 2:1. Parasite cultures were treated with plasma gel to obtain erythrocytes with 15% parasitaemia of mixed stages from late rings to mid trophozoites. Subcultures were incubated with labelled haptoglobin or albumin (5 mg mL^{-1}) for 2 hours at 4°C and 37°C. Erythrocytes (either unfixed or fixed with 1:1 acetone/methanol) were examined under fluorescence microscope. Indirect immunofluorescence was performed on haptoglobin-treated parasitised cells fixed with 1:1 methanol/acetone. Primary antibodies used were rabbit anti-human haptoglobin (Sigma), rabbit anti-PfEMP1 'var c' (courtesy of Professor Dr Brian Cooke, Monash University, Victoria, Australia), monoclonal anti-KAHRP 'Mab 89' (courtesy of Professor Diane Taylor, Georgetown University, Washington, USA). Secondary antibodies used were swine anti-rabbit IgG-FITC (Dako) and goat anti-mouse F(ab')2-FITC (Dako). The nuclear stain 4',6-Diamidino-2-phenylindole (DAPI) (Molecular probes) was used to detect parasitised cells.

3. Results

Using flow cytometric analysis it was found that addition of 5 mg mL^{-1} pooled haptoglobin to cultures of early trophozoites (18-24 hours after invasion) resulted in reduced parasitaemias following one re-invasion cycle (Fig.1a). The inhibitory effect of haptoglobin was found to occur with various parasite lines, viz: Muz 37.4, 3D7, Muz 37, 1776, 1776/C10 (data not shown), none of the lines tested being insensitive to haptoglobin. Addition of haptoglobin to parasite cultures for 8 hour periods

throughout the 48 hour life cycle demonstrated that it was necessary for the haptoglobin to be present 16-24 hours after sorbitol treatment when the parasites were a range of 16-30 hours post-invasion, i.e. at the late ring and early trophozoite stages of development (Fig 1b). Measurement of tritiated hypoxanthine uptake showed that nucleic acid production was reduced 15 hours after addition of the haptoglobin, i.e. production was reduced during the trophozoite maturation phase, 31-37 hours post-invasion (Fig. 2). Although the parasites were sensitive to haptoglobin added to cultures at the late ring-early trophozoite stage, no changes in parasite morphology were visible by light microscopy until 24 hours after the addition of haptoglobin. At this time the majority of parasites retained the appearance of mid-stage trophozoites (Fig 3B), unlike control parasites which had developed into early schizonts (Fig3A). No decrease in parasitaemia was detected at this time by either light or electron microscopy. In addition, electron microscopy also identified a number of aggregations of electron dense material associated with Maurer's clefts, present in 48% of infected erythrocytes in the treated samples (Fig 3B insert) compared with <1% in untreated controls (Fig. 3A insert). Thus microscopy demonstrated that the presence of haptoglobin resulted in a failure of parasite maturation and re-invasion.

Biotin-labelling of haptoglobin suggested that it was present in the whole erythrocyte fraction following incubation at 4°C and 37°C. It was also detected in the parasite pellet, with a stronger band being found after incubation at 37°C. There was no anti-glycophorin A and a trace of albumin detected in parasite pellets (data not shown). However, immunofluorescence showed that haptoglobin binds to the surface of a small population of erythrocytes which are in the process of rupturing and extracellular parasites (albumin also binds to the latter). No binding was evident in

those erythrocytes and parasites which exhibited normal morphology. Immunofluorescence also showed that the material detected in Maurer's cleft by EM was not haptoglobin and was unlikely to be PfEMP1 or KAHRP since both these parasite proteins were distributed normally within erythrocytes at various time points during the parasites life cycle in haptoglobin-treated and control cultures.

When the various haptoglobin phenotypes were added to cultures it was found that inhibition occurred in the order 1-1 > 2-2 > 2-1, inhibition by Hp2-1 only being detectable by tritiated hypoxanthine uptake (Fig. 4), not by flow cytometry (data not shown).

4. Discussion

Our results show that haptoglobin has a deleterious effect on the growth of *P. falciparum in vitro* at concentrations observed physiologically during the acute phase response [17], [18]. Evidence to support a role for an anti-parasitic effect of haptoglobin *in vivo* comes from experiments demonstrating that peak parasitaemia and peak parasite burden were greater in haptoglobin knock-out mice infected with *P. berghei* and *P. chabaudi* compared to wild type mice [11].

The light microscopic changes in haptoglobin-treated parasites are not typical of those seen in the so called 'crisis forms' which occur when parasites are cultured in some sera from non-immune individuals from malarious areas, possibly due to the presence of cytokines such as TNFα [19]. Crisis form parasites fail to develop properly within the erythrocyte, appearing as atypical rings or young trophozoites devoid of haemozoin [20], whereas the haptoglobin-treated parasites are affected during the trophozoite maturation phase.

We are currently investigating the mechanism of *P. falciparum* toxicity of haptoglobin. Initially it was thought that haptoglobin might act in the intracellular environment; whereas haptoglobin is an antioxidant and neutralises the potentially toxic effects of haemoglobin at physiological pH, at pH < 5.5 haptoglobin enhances the peroxidase activity of haemoglobin [21]. It was hypothesised that haptoglobin may be internalised by the intra-erythrocytic parasite and become toxic once within the acid environment of the food vacuole. However, we were unable to demonstrate internalisation of FITC-labelled haptoglobin within the erythrocyte or parasite (our results also show that investigations using biotin-labelling should always been interpreted with caution). In addition we have found that lysosmotrophic agents do not affect the toxic effects of haptoglobin (unpublished observations).

Thus we propose that haptoglobin affects the extracellular environment in a manner that is deleterious to the parasite. Others have shown that haptoglobin 2-2 and 2-1 cause agglutination of T4 *Streptococcus pyogenes* [7] but we have shown that haptoglobin does not cause agglutination of parasitised cells (unpublished observations). Haptoglobin is known to have a bacteriostatic effect on *Escherischia coli* due to its ability to bind hemoglobin [22] and thus it may affect the uptake of nutrients by *P. falciparum* in an analogous manner. *P. falciparum* produces complex membranous structures within the host cell cytoplasm, including flattened lamaellae called Maurer's clefts which are implicated in the transport of parasite proteins to the erythrocyte plasma membrane and submembrane skeleton [23]. The appearance of electron-dense material (at present unidentified) in Maurer's clefts following incubation with haptoglobin suggests that it interferes with protein trafficking. This in

turn may affect the development of the parasite and on current evidence would appear to be the mechanism of toxicity.

Our results also showed that the toxic effect of haptoglobin is phenotype-dependent; in descending order of toxicity: Hp1-1 > Hp2-2 > Hp2-1, therefore, it might be expected that there may be selection pressure for Hp 1-1 in malaria-endemic areas. The Hp^2 allele is estimated to have originated in India approximately 2 million years ago and has since spread over the world under a strong genetic pressure. It has been suggested that the balance between Hp^1 and Hp^2 alleles is due to the fact that individuals of Hp1-1 phenotype are able to handle free haemoglobin more efficiently but are likely to have reduced antibody responses due to the greater ability of Hp1-1 to bind CD22 and inhibit lymphocyte transformation [10]. The association between malaria endemicity and haptoglobin alleles is controversial. Phenotype-dependent variability in the efficiency of malaria toxicity and of haemoglobin clearance are likely to influence allele frequencies in malaria endemic areas. Reports on the association between malaria and haptoglobin phenotypes have yielded conflicting results; some authors have suggested that the phenotype Hp1-1 is associated with susceptibility to falciparum malaria and with the development of severe complications [24]; [25], whereas others found an association between Hp1-1 and malaria resistance [26] or no association [27].

The role which haptoglobin may play in *in vivo* protection against malaria is likely to be complex. Due to its role in haemoglobin clearance, the presence of haptoglobin is of importance during the course of an episode of malaria. In individuals mounting an acute phase response to *P. falciparum*, haptoglobin may initially be increased to malaria-toxic levels. Later during the course of an infection the haemolysis which accompanies chronic, subclinical malaria reduces plasma

concentrations of haptoglobin and thus levels could fall below those at which the malaria toxic effect occurs. We do not know whether *in vitro* toxic levels are the same as those necessary to kill the parasite *in vivo*. Trophozoites adhere in the deep vasculature and cannot be seen in the circulation. Thus, the morphological changes we have observed *in vitro* cannot be checked in the human host.

Based on our in vitro observations and the in vivo experiment in knock out mice, we propose that the acute phase protein, haptoglobin may have a role in innate defence against malaria. Further investigation is necessary in order to elucidate the mechanism and phenotype-dependence of haptoglobin malaria toxicity.

Acknowledgements

We would like to thank Professor Steve Hajduk (MBL, Woods Hole, Massachusetts, USA) for his help and advice. This research was funded by the Wellcome Trust.

References

[1] Dobryszycka W. Biological functions of haptoglobin - new pieces to an old puzzle. Eur J Clin Chem. Clin Biochem 1997; 35: 647-54

[2] Smithies O. Zone electrophoresis in starch gels: group variations in the serum proteins of normal human adults. Biochem J 1955; 61: 629-41

[3] Lim YK, Jenner A, bin Ali A, Wang Y, Hsu Stephen IH, Chong Siew M, *et al.* Haptoglobin reduces renal oxidative DNA and tissue damage during phenylhydrazine-induced hemolysis. Kidney Int 2000; 58: 1033-44.

[4] Delanghe J, Allcock K, Langlois M, Claeys L, DeBuyzere M. Fast determination of haptoglobin phenotype and calculation of hemoglobin binding capacity using high pressure gel permeation chromatography. Clinica Chimica Acta 2000; 291: 43-51

[5] Saeed SA, Mahmood F, Shah BH, Gilani AH. The inhibition of prostaglandin biosynthesis by human haptoglobin and its relationship with haemoglobin binding. Biochem Soc Transactions 1997; 25: S618-S

[6] Cid MC, Grant DS, Hoffman GS, Auerbach R, Fauci AS, Kleinman HK. Identification of haptoglobin as an angiogenic factor in sera from patients with systemic vasculitis. J Clin Invest 1993; 91: 977-85

[7] Ouyang J, Delanghe J, Baeyens WRG, Langlois M. Application of Western-blotting technique with chemiluminescence imaging to the study of haptoglobin type and haptoglobin complexes. Analytica Chimica Acta 1998. 362, 113-20

[8] Baseler MW, Burrell R. Purification of haptoglobin and its effects on lymphocyte and alveolar macrophage responses. Inflammation 1983; 7: 387-400

[9] Arredouani M, Matthijs P, Van Hoeyveld E, Kasran A, Baumann H, Ceuppens JL et al. Haptoglobin directly affects T cells and suppresses T helper cell type 2 cytokine release. Immunol 2003; 108: 144-51

[10] Langlois MR, Delanghe JR. Biological and Clinical Significance of Haptoglobin Polymorphism in Humans. Clin Chem 1996; 42: 1589-600

[11] Hunt NH, Driussi C, Sai-Kiang L. Haptoglobin and malaria. Redox Report : Commun Free Radical Res 2001; 6: 389-92

[12] Trager W, Jenson JB. Human malaria parasites in continuous culture. Science 1976; 193: 674-5

[13] Day KP, Karamalis F, Thompson J, Barnes DA, Peterson C, Brown H, *et al.* Genes necessary for expression of a virulence determinant and for transmission of *Plasmodium falciparum* are located on a 0.3 Megabase region of chromosome 9. Proc Nat Acad Sci USA 1993; 90: 8292-6

[14] Pasvol G, Wilson RJM, Smalley ME, Brown J. Separation of viable schizont-infected red cells of *Plasmodium falciparum* from human blood. Annal Trop Med Parasitol 1978; 72: 87-8

[15] Lambros C, Vanderburg JP. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J Parasitol 1979; 65: 113-39

[16] Piper KP, Roberts DJ, Day KP. *Plasmodium falciparum*: analysis of the antibody specificity to the surface of the trophozoite-infected erythrocyte. Exp Parasitol 1999; 91:161-9

[17] Ebersole JL, Machen RL, Steffen MJ, Willmann DE. Systemic acute-phase reactants, C-reactive protein and haptoglobin, in adult periodontitis. Clin Exp Immunol 1997; 107: 347-52

[18] Buttenschoen K, Fleischmann W, Haupt U, Kinzl L, Buttenschoen DC. Translocation of endotoxin and acute-phase proteins in malleolar fractures. J Trauma 2000; 48: 241-5

[19] Phillips S. Effector mechanisms against asexual erythrocytic stages of *Plasmodium*. Immunol Lett1994; 41:109-14

[20] Jensen JB, Boland MT, Akood M. Induction of crisis forms in cultured *Plasmodium falciparum* with human immune serum from Sudan. Science 1982; 216: 1230-3

[21] Tomlinson S, Raper J. The lysis of *Trypanosoma brucei brucei* by human Serum. Nature Biotechnol 1996; 14: 717-21

[22] Blisnick T, Morales Betoulle ME, Barale JC, *et al.* Pfsbp1, a Maurer's cleft *Plasmodium falciparum* protein, is associated with the erythrocyte skeleton. Mol Biochem Parasitol 2000; 111: 107-21

[23] Eaton J W, Brandt P, Mahoney JR, Lee JT Jr. Haptoglobin: a natural bacteriostat Science 1982; 215: 691-3

[24] Elbashir MI. Association of the haptoglobin phenotype (1-1) with *falciparum* in Sudan. MIM African Malaria Conference 1999, C-58 (Abstract)

[25] Quaye IKE, Ekuban FA, Goka BQ, Abadayeri V, Kurtzhals JAL, Gyan B *et al.* Haptoglobin 1-1 is associated with susceptibility to severe *Plasmodium falciparum* malaria. Trans Royal Soc Trop Med Hyg 2000; 94: 216-9

[26] Bottini N, Ronchetti MP, Gloria Bottini F, Fontana L. Malaria as a possible evolutionary cause of allergy. Allergy 1999; 54: 188-9

[27] Aucan C, Walley AJ, Greenwood BM, Hill AV. Haptoglobin genotypes are not associated with resistance to severe malaria in The Gambia. Trans Royal Soc Trop Med Hyg 2002; 96: 327-8

Figure 1a

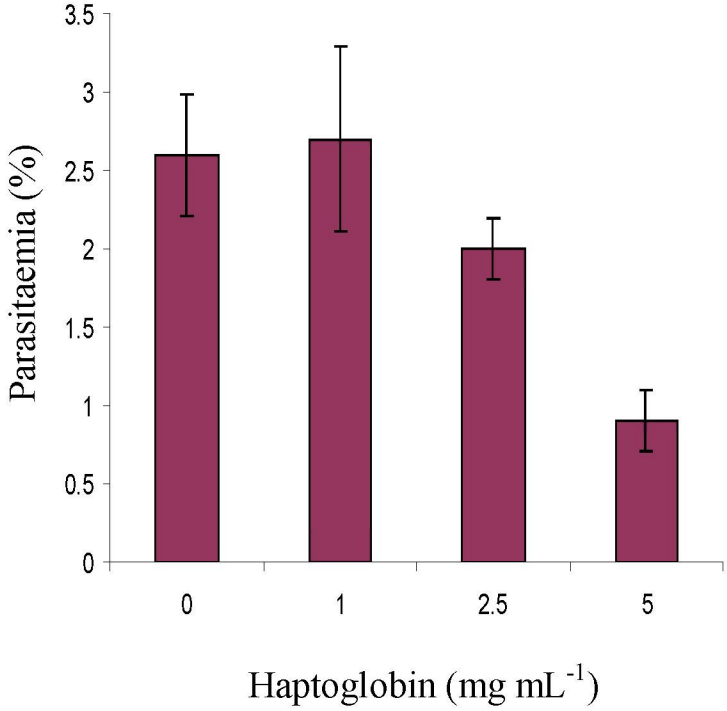

Figure 1b

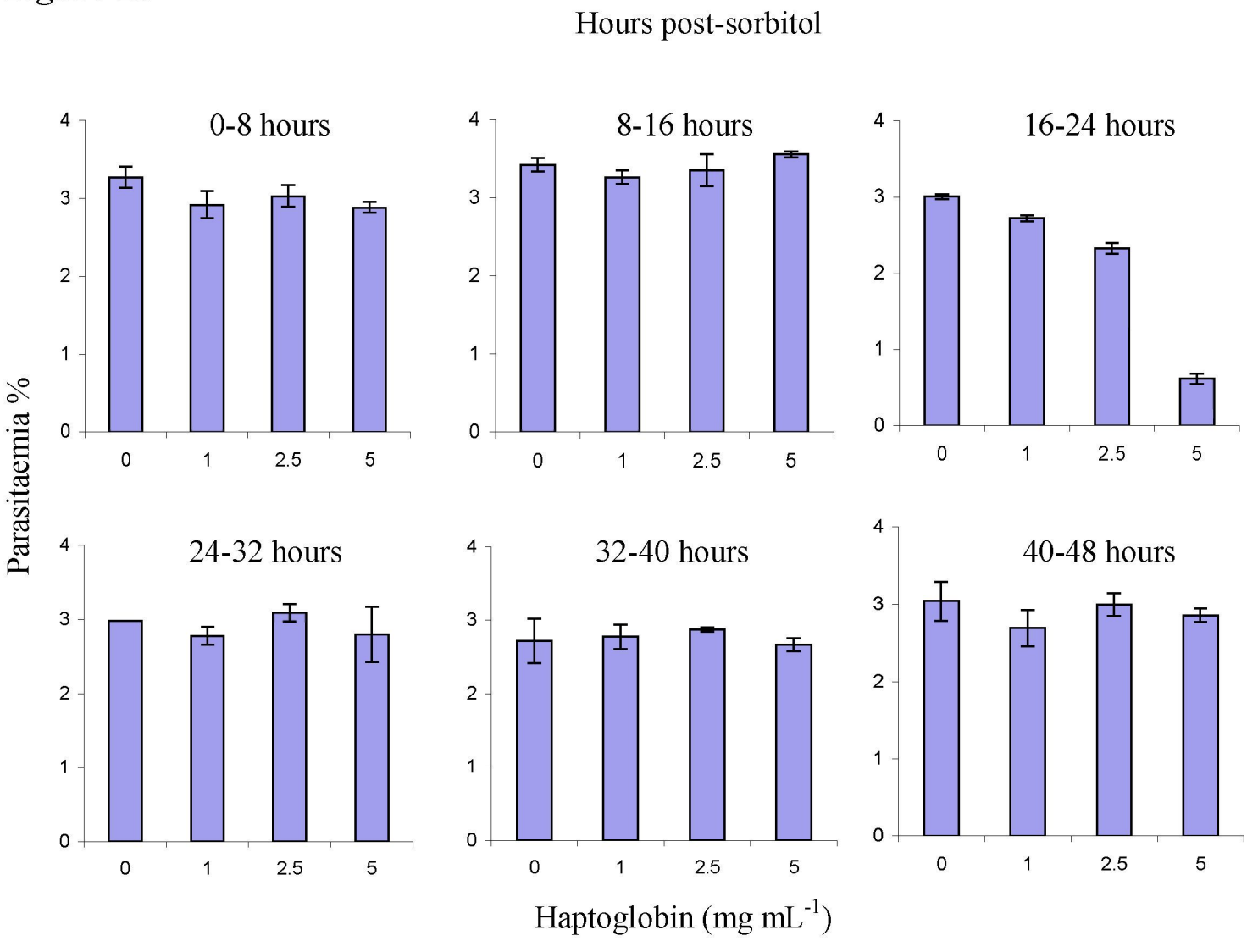

Figure 2

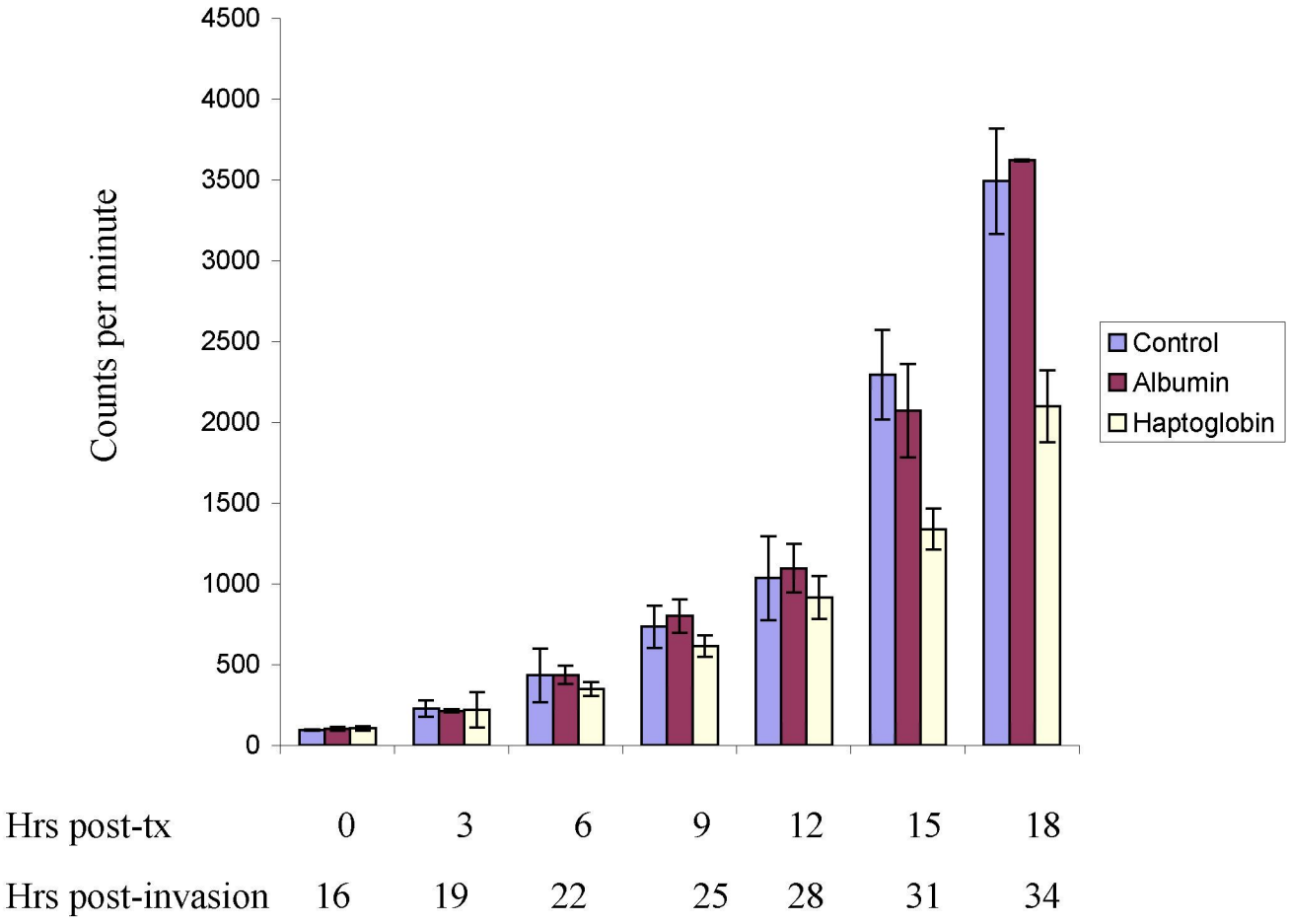
Counts per minute
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Control
Albumin
Haptoglobin
Hrs post-tx 0 3 6 9 12 15 18
Hrs post-invasion 16 19 22 25 28 31 34

Figure 3

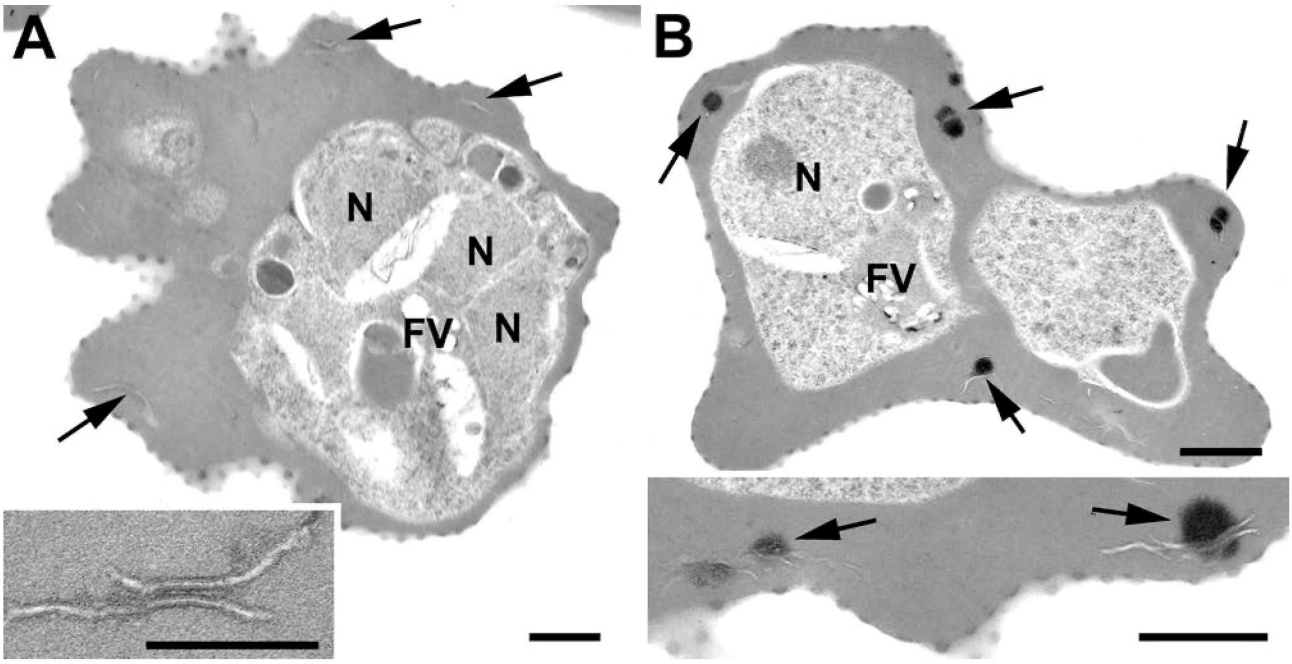

Figure 4

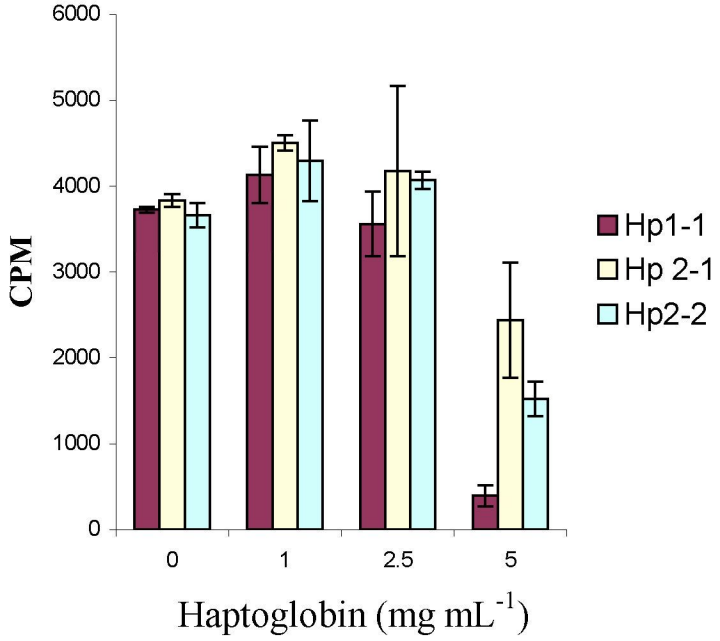
6000
5000
4000
3000
2000
1000
0
CPM
0
1
2.5
5
Haptoglobin (mg mL-1)
Hp1-1
Hp 2-1
Hp2-2

Figure legends

Fig. 1:Effects of haptoglobin on parasite replication

Parasites (1% parasitaemia, late rings) were grown in medium containing various concentrations of haptoglobin (pooled Hp1-1, Hp2-1 and Hp2-2). Following erythrocyte re-invasion, parasitaemias were determined by flow cytometry. Figure 1a shows the effect of adding haptoglobin to parasite cultures 18-24 hours after merozoite invasion. Figure 1b shows the effect of adding haptoglobin for 8 hours periods at various time points throughout the 48 hour lifecycle.

Fig. 2. Effect of haptoglobin on parasite nucleic acid synthesis

Parasites (1% parasitaemia, late rings) were grown in medium containing ^{3}H-hypoxanthine. Haptoglobin (pooled phenotypes) was added at a final concentration of 5 mg mL^{-1}. Control cultures were grown in medium without any addition protein 'control' or with 5 mg mL^{-1} human albumin. At various time points following this treatment (Hrs post-tx), parasite nucleic acid synthesis was determined by harvesting cells onto filter paper and measuring scintillation counts per minute. Parasites were synchronous to within 6 hours; the minimum time post-invasion is shown on the horizontal axis (Hrs post-invasion).

Fig. 3. Effect of haptoglobin on parasite structure

Parasites (3% parasitaemia, late rings) were grown in control medium (A) or medium containing 5 mg mL^{-1} haptoglobin (phenotype Hp1-1)(B). After 24 hours, parasites were fixed and examined by electron microscopy. A. Control sample showing an

erythrocyte containing an early multinucleate schizont. Note the indistinct Maurer's clefts around the periphery of the erythrocyte (arrows). N – nucleus; FV- food vacuole. Insert. Detail of the periphery of an erythrocyte showing the structure of the Maurer's clefts.

B. Treated sample showing an erythrocyte containing two early/mid trophozoites. Note the electron dense material associated with the Maurer's clefts (arrows). N – nucleus; FV – food vacuole. Insert. Detail of the Maurer's clefts in which electron dense material is associated with the outer surface (arrows). Bars represent 0.5μm.

Fig 4. Effect of haptoglobin phenotype on parasite nucleic acid synthesis and replication

Parasites (1% parasitaemia, late rings) were grown in medium containing ^{3}H-hypoxanthine and various concentrations of haptoglobin (phenotypes Hp1-1, Hp2-1 and Hp2-2). After 24 hours, parasite nucleic acid synthesis was determined by harvesting cells onto filter paper and measuring scintillation counts per minute (CPM). Haptoglobin reduced parasite nucleic acid synthesis in a dose-dependent manner, the phenotype order of efficacy being Hp1-1 > Hp2-2 > Hp2-1.