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Cytoadherence and virulence - the case of *Plasmodium knowlesi* malaria

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Abstract

Background: Cytoadherence of infected red blood cells to brain endothelium is causally implicated in malarial coma, one of the severe manifestations of falciparum malaria. Cytoadherence is mediated by specific binding of variant parasite antigens, expressed on the surface of infected erythrocytes, to endothelial receptors including, ICAM-1, VCAM and CD36. In fatal cases of severe falciparum malaria with coma, blood vessels in the brain are characteristically congested with infected erythrocytes. Brain sections from a fatal case of knowlesi malaria, but without coma, were similarly congested with infected erythrocytes. The objective of this study was to determine the binding phenotype of *Plasmodium knowlesi* infected human erythrocytes to recombinant human ICAM-1, VCAM and CD36.

Methods: Five patients with PCR-confirmed *P. knowlesi* malaria were recruited into the study with consent between April and August 2010. Pre-treatment venous blood was washed and cultured *ex vivo* to increase the proportion of schizont-infected erythrocytes. Cultured blood was seeded into Petri dishes with triplicate areas coated with ICAM-1, VCAM and CD36. Following incubation at 37°C for one hour the dishes were washed and the number of infected erythrocytes bound/mm^2 to PBS control areas and to recombinant human ICAM-1 VCAM and CD36 coated areas were recorded. Each assay was performed in duplicate. Assay performance was monitored with the *Plasmodium falciparum* clone HB3.

Results: Blood samples were cultured *ex vivo* for up to 14.5 h (mean 11.3 ± 1.9 h) to increase the relative proportion of mature trophozoite and schizont-infected red blood cells to at least 50% (mean 65.8 ± 17.51%). Three (60%) isolates bound significantly to ICAM-1 and VCAM, one (20%) isolate bound to VCAM and none of the five bound significantly to CD36.

Conclusions: *Plasmodium knowlesi* infected erythrocytes from human subjects bind in a specific but variable manner to the inducible endothelial receptors ICAM-1 and VCAM. Binding to the constitutively-expressed endothelial receptor CD36 was not detected. Further work will be required to define the pathological consequences of these interactions.

Keywords: *P. knowlesi*, Cytoadherence, SICAvar, ICAM-1, VCAM, CD36, Malaria, Coma

Background

Coma is one of the manifestations of *Plasmodium falciparum* malaria in children and adults [1,2] and it carries a poor prognosis. The accumulation of cytoadherent parasitized erythrocytes in post-capillary venules of the brain is strongly causally implicated in precipitating malarial coma [3-5]. Adherence to brain and other endothelial surfaces is mediated by the expression of variant parasite-derived proteins (Pf EMP1 *var* family) on the *P. falciparum* infected erythrocyte surface [6]. PfEMP1 proteins predominantly bind to CD36, but also to inducible Intercellular Adhesion Molecule 1 (ICAM-1) [7-10]. Binding to up-regulated ICAM-1 is particularly important in cytoadherence to brain endothelium because CD36 is not expressed in this endothelial compartment [8,9].

Malarial coma is rare in other infections by the human host-adapted *Plasmodium* species and coma has not been a feature of severe and fatal zoonotic *Plasmodium*

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knowlesi malaria [11-13]. However, *post mortem* examination of a fatal case of severe knowlesi malaria without coma showed brain capillaries and venules congested with infected erythrocytes [14]. Expressed parasite variant surface antigens had been described in experimental *P. knowlesi* infections of rhesus monkeys before PfEMP1 was identified in *P. falciparum* [15]. *Plasmodium knowlesi* surface proteins were named Schizont-Infected Cell Agglutination Antigens (SICA) and are encoded by the SICA*var* gene family [16]. Although distantly related, SICAvar proteins share binding signature motifs with PfEMP1 proteins [17]. The remarkable histological similarity between brain sections from fatal *P. knowlesi* malaria and fatal cases of severe falciparum malaria with coma [14,18], particularly the accumulation of infected erythrocytes in brain microvasculature, led to the design of this study to test the binding characteristics of *P. knowlesi* isolates from patients [9].

Methods

Patient recruitment

Patients with malaria admitted to Hospital Sarikei in Sarawak, Malaysian Borneo were recruited, with informed consent, into this study between April and August 2010. The study was approved by the Malaysian Ministry of Health Medical Research and Ethics Committee. Infecting species was confirmed by *Plasmodium* species-specific nested-PCR assays [19] and only patients with single species infections were retained in the study.

Blood collection and ex vivo parasite development

Approximately 2.5 mL of pre-treatment venous blood from each patient was collected into EDTA. RPMI 1640 medium supplemented with 20 mM D-glucose, 25 mM HEPES, 25 mg/ml gentamicin sulphate, 15% human AB plasma with 0.2 mM hypoxanthine was used for parasite culture. Gently washed loosely packed cells from each patient were re-suspended in culture medium to approximately 5% haematocrit and cultured at 37°C under 5% O_2, 5% CO_2 and 90% N_2. Thin blood film microscopy was used to follow parasite development until at least half the parasites had developed into late trophozoites (parasites with dense cytoplasm and undivided nuclear chromatin mass) and schizonts (at least three divided nuclear chromatin masses with pigment granules). See Figure 1a and 1b.

Static protein binding assays

A method adapted from McCormick *et al.* [9] to test the ability of infected erythrocytes to bind to purified recombinant human Fc chimera ICAM-1, VCAM, and CD36 (R&D Systems, Minneapolis, USA) was used. Three identical areas of each Petri dish (60 mm diameter, product code 351007, Becton, Dickenson and Company, NJ, USA) were treated with 2 μl aliquots of purified ICAM-1, VCAM, CD36, each at 100 μg/mL. Control areas were treated with phosphate buffered saline (PBS) and three marked areas were left untreated. The dishes were incubated in a humid chamber at 37°C for two hours before aspirating off excess protein and blocking all areas with 1% w/v bovine serum albumin in PBS for 2 h at 37°C. The blocking solution was removed by gentle pipetting. *Ex vivo* matured infected erythrocyte cultures were added to 3 ml warmed binding buffer (RPMI 1640 media supplemented with D-glucose) to a final haematocrit of 3%. Each protein and the PBS control were represented in triplicate per dish and duplicate dishes were seeded per patient isolate. Therefore there
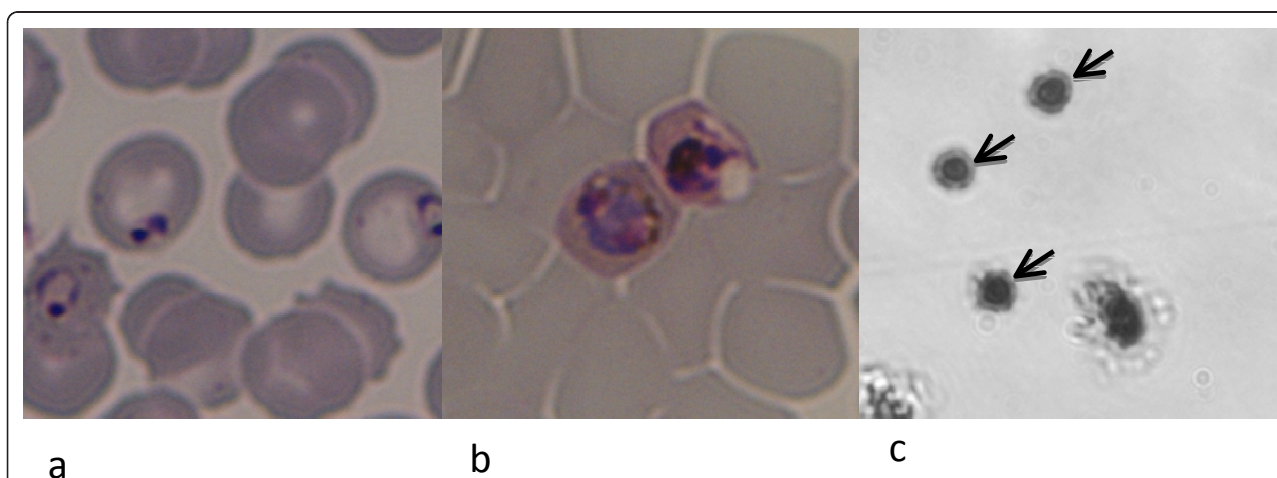

Figure 1 *Plasmodium knowlesi* static binding assay. On admission to the study this patient had predominantly ring (immature trophozoite) stage parasites (**a**). Following a period of *in vitro* culture the parasites matured, in this case to late trophozoite stages, as required for the static binding assay (**b**). Infected erythrocytes bound to an ICAM-1 coated area of the assay dish are marked with arrows (**c**).

were six replicate areas per protein or PBS control for each patient sample assayed. Dishes were seeded with 1.5 ml of the prepared *ex vivo* cultured cell suspension per patient isolate and a third assay dish with *P. falciparum* clone HB3 as an assay performance control.

The dishes were incubated at 37°C for 1 h, with gentle mixing at 10 min intervals. Unbound cells were removed by gentle washing seven times with RPMI 1640 supplemented with D-glucose. Bound cells were fixed with 1% v/v gluteraldehyde for 1 h and stained with 10% Giemsa. For example see Figure 1c. Using an inverted light microscope at × 300 magnification images from ten consecutive non-overlapping fields for each protein and PBS treated area (six areas each per patient sample) were captured. This was equivalent to an area of 0.135 mm^2. The number of bound infected cells/0.135 mm^2/ protein or control were counted. The results were expressed as the number of infected cells (IE) bound/ mm^2 to each of the proteins or control [IE/mm^2 = (1/ 0.0135) × mean number of bound IE per field]. Significant binding of *P. falciparum* clone HB3 to all three test proteins compared with PBS was required in the assay performance control to validate the results for each patient isolate. Purified protein binding assays on

primary field isolates are quantifiable within but not between isolates because of variability in parasitaemia. Significant binding to each protein compared with binding to PBS was determined using the Mann-Whitney *U* Test (Graphpad PRISM version 4.0a San Diego California, USA).

Results

Five patients with PCR confirmed single species *P. knowlesi* infections were recruited into the study. Parasitaemia ranged from 0.4 to 7%. Clinical and parasitological data are summarised in Table 1. Apart from patient (P0009) with high parasitaemia and renal failure the patients had acute uncomplicated malaria. Blood samples were cultured *ex vivo* for up to 14.5 h (mean 11.3 ± 1.9 h) to allow parasite maturation and increase the relative proportion of mature trophozoite and schizont-infected red blood cells to at least 50% (mean 65.8 ± 17.51%) of all parasite stages present in preparation for binding assays (Table 1).

Binding to ICAM-1, VCAM and CD36

Three (60%) isolates bound significantly to ICAM-1 and VCAM. One (20%) isolate bound significantly to VCAM

Table 1 Patient demographic, clinical and laboratory features including *ex vivo* parasite development in preparation for static binding assays
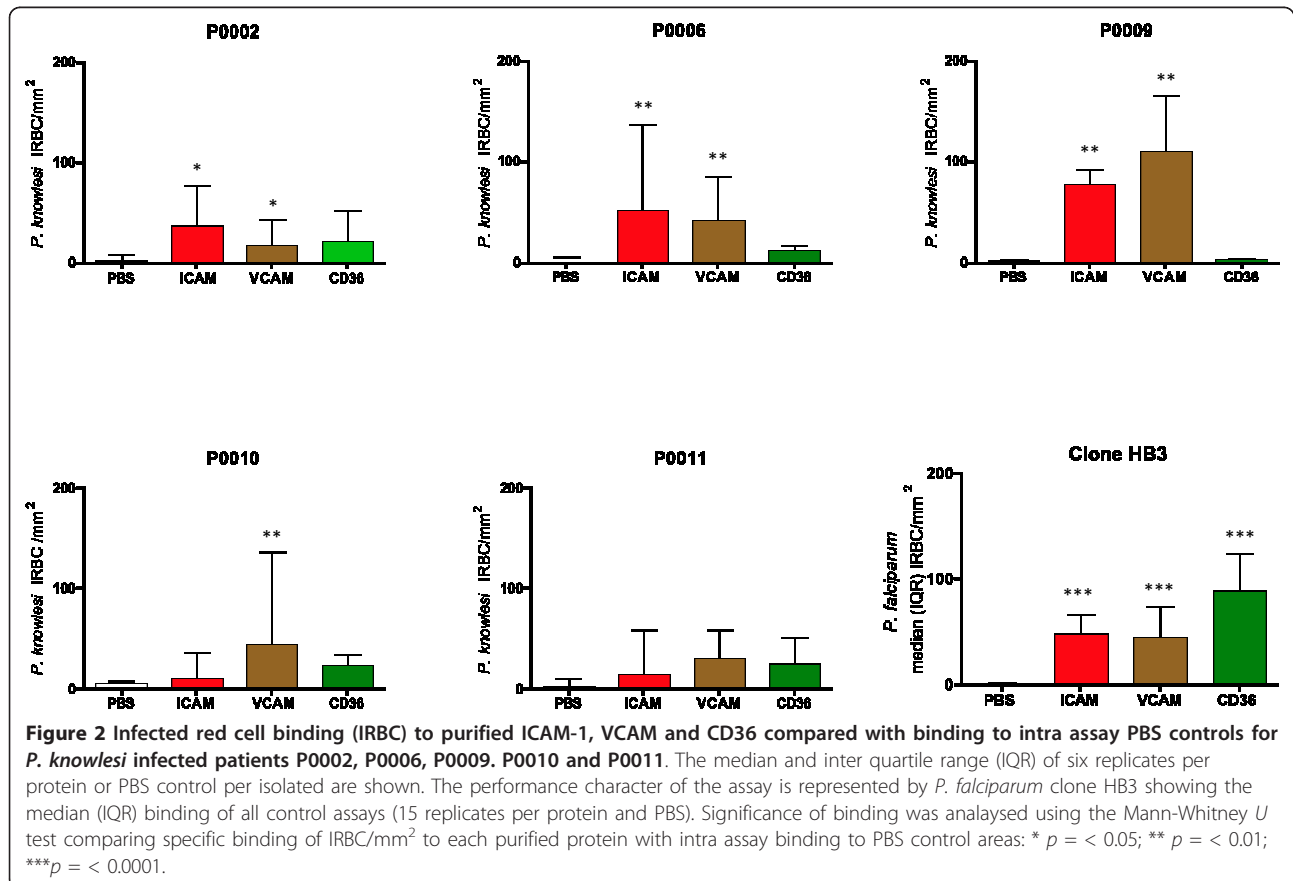
	Patient details				
Study reference	P0002	P0006	P0009	P0010	P0011
Age	16	47	28	27	37
Sex	M	F	M	M	M
% Parasitaemia	0.4	0.8	7	0.8	1.3
Fever duration (days)	7	5	6	5	4
Axillary Temp°C	37.5	38.5	39.2	38.7	38
Mean Art BP mmHg	79	96	70	86	77
Pulse per min	62	96	96	103	94
Haemoglobin g/dL	13.4	13.2	12.3	14.1	12.8
Platelets/uL	93,000	106,000	19,000	39,000	65,000
White cells/uL	6600	6700	6900	7500	4600
Sodium mmol/L	133	131	120	132	131
Potassium mmol/L	3.7	4.9	3.6	3.3	3.9
Chloride mmol/L	100	99	92	101	100
Total bilirubin umol/L	20.6	15.7	38.4	9.7	48.9
Conjugated bilirubin umol/L	5	12.8	23	4	32.6
Alkaline phosphatase U/L	100	62	87	131	233
Alanine aminotransferase U/L	28	21	43	35	68
Asparagine aminotransferase UL	17	30	118	23	22
Blood urea mmol/L	4.4	5.7	44.7	6	8.8
Serum creatinine umol/L	-	78	600	59	133
	Static binding assay details				
% Mature Troph/Schizonts before *ex vivo* culture	25	40	17	10	10
Hours *ex-vivo* culture	10	14.5	10	10	12
% Mature Troph/Schizonts at start of assay	75	50	91	63	50

and one isolate did not bind significantly to ICAM-1, VCAM or CD36 (Figure 2). None of the *P. knowlesi* isolates tested showed significant binding to CD36 (Figure 2). The performance of each binding assay was monitored using *P. falciparum* clone HB3. Clone HB3 significantly bound to ICAM-1, VCAM and CD36 in all assay plates and clone HB3 binding characteristics are summarised in Figure 2.

Discussion

Plasmodium knowlesi infected erythrocytes from human infections bind in a specific but variable manner to the human endothelial cell receptors ICAM-1 and VCAM but not to CD36. Specific binding of infected erythrocytes to endothelial cell receptors is responsible for cytoadherence and, therefore, sequestration of late trophozoite and schizont infected erythrocytes from the peripheral blood circulation in *P. falciparum* malaria [6]. With few exceptions only immature trophozoite stages of *P. falciparum* infected erythrocytes are found in the circulation of patients with acute uncomplicated falciparum malaria. *Plasmodium falciparum* PfEMP1 proteins predominantly bind to CD36, a constitutively expressed scavenger pattern recognition protein, and

variably to ICAM-1 and other up-regulated endothelial cell receptors [8,9,20,21]. CD36 is not expressed on areas of brain endothelium where cytoadherence occurs [22] and binding to ICAM-1 on brain endothelium is implicated in the pathophysiology of severe falciparum malaria with coma [8,9,20]. Particular expression of PfEMP1 variants, with differential ability to bind ICAM-1, has been demonstrated in malaria patients with coma [22,23]. Parasite sequestration through cytoadherence in *P. falciparum* infections can be organ specific but it is not clear yet whether this equates with virulence rather than with available binding sites and parasite binding affinity [20,21,24,25]. There is some data to suggest stratification of the *var* type early in infection and it has been suggested that cytoadherence to available receptors is responsible for this [26]. Mature stage parasites are observed in the circulation of all other types of malaria of humans, including *P. knowlesi* malaria. However, this does not exclude the possibility of a degree of parasite sequestration by specific binding to endothelial cell receptors or by other means. ICAM-1 is expressed in low copy number in resting endothelium and is up-regulated in inflammation and infection [25,27,28] including severe malaria with coma [7]. In falciparum

Figure 2 Infected red cell binding (IRBC) to purified ICAM-1, VCAM and CD36 compared with binding to intra assay PBS controls for *P. knowlesi* infected patients P0002, P0006, P0009. P0010 and P0011. The median and inter quartile range (IQR) of six replicates per protein or PBS control per isolated are shown. The performance character of the assay is represented by *P. falciparum* clone HB3 showing the median (IQR) binding of all control assays (15 replicates per protein and PBS). Significance of binding was analaysed using the Mann-Whitney *U* test comparing specific binding of IRBC/mm^2 to each purified protein with intra assay binding to PBS control areas: * p = < 0.05; ** p = < 0.01; ***p = < 0.0001.

malaria TNF has been implicated in ICAM-1 up-regulation although infected red blood cells alone are sufficient to produce this effect [25]. *Plasmodium falciparum* isolates from patients with differing degrees of disease severity show endothelial receptor binding diversity [29] with binding to ICAM-1 highest and most robust in *P. falciparum* isolates from patients with severe malaria with coma [22,29]. *Plasmodium knowlesi* proteins expressed on infected erythrocytes do not appear to bind to CD36 is this study. This lack of adhesion in a robust manner to constitutively expressed abundant endothelial receptors, such as CD36, would explain the presence of mature stage parasites in the circulation when inducible target receptors are not up-regulated. Significant but variable binding of *P. knowlesi* infected erythrocytes from human infections to the inducible endothelial receptors ICAM-1 and VCAM was demonstrated here. This result suggests that, if up-regulated on brain endothelium, *P. knowlesi* infected erythrocytes could potentially cytoadhere to ICAM-1 in that compartment. Notwithstanding this possibility, immunohistochemistry failed to detect ICAM-1 on the endothelial surfaces in parasite congested brain sections of a fatal case of severe knowlesi malaria without coma [14]. There may be technical explanations for the failure to detect ICAM-1 including limited available sections, delayed post-mortem sampling and loss of tissue integrity. It is also possible that ICAM-1 is not induced on brain endothelium in knowlesi infections and that specific binding *ex vivo* is not associated with parasite virulence in this species. The intense accumulation of infected erythrocytes observed in brain sections in fatal knowlesi malaria may have occurred through processes other than specific cytoadherence, for example infected cell agglutination [15]. This study and clinical descriptions of severe and fatal knowlesi malaria suggest that up-regulation of ICAM-1 observed in *P. falciparum* infections is less evident in *P. knowlesi* malaria and that direct or indirect induction of ICAM-1 may be a defining Plasmodium species-specific virulence factor in severe malaria with coma.

Conclusions
In summary, *P. knowlesi* infected erythrocytes from human subjects can bind to the inducible endothelial receptors ICAM-1 and VCAM. Further work will be required to define the pathological implication of these interactions.

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Authors' contributions
The study was conceived by JCS and designed by JCS, AGC, SK and BS. The assays were performed by FAF with field support from AS, MAH and LCW. The manuscript was prepared by JCS, ACG, and SK. All authors had the opportunity to read and approve the manuscript.

Competing interests
The authors declare that they have no competing interests.

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