Characterization of Ligands Mediating Adherence of *Plasmodium falciparum* Infected Erythrocytes to CD36 and ICAM-1

by

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ABSTRACT

All models of cerebral malaria pathogenesis involve sequestration; the unique ability of *Plasmodium falciparum* IE to bind to vascular endothelium. Thus central to understanding cerebral malaria is the elucidation of the molecular interactions of sequestration.

In these studies, detailed time course experiments were performed to help define the roles of the ligands PfEMP1 and band 3 neoantigens and receptors CD36 and ICAM-1. The ability to bind to CD36 and ICAM-1 is an off/on event closely linked to the ring to trophozoite transition.

Band 3 neoantigens 1F4, 3H3 are surface expressed beginning at 12 hours and 1C4 is present on the surface coincident with the onset of binding. These neoantigens are coincidentally present on individual IE, with unique patterns of distribution.

PfEMP1 is present on the surface of fixed and live infected erythrocytes coincident with the onset of binding. Confocal microscopy revealed that the protein is synthesized beginning 8 hours before binding in the parasite cytoplasm. In agreement with this, is the upregualtion of *var* gene transcription in the late ring stage demonstrated by RT-PCR.

Dual labeling studies demonstrate that the two temporally relevant ligands, PfEMP1 and 1C4, are both found on single IEs, but appear to have distinct distributions, suggesting that they may function independently in promoting IE sequestration.

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INTRODUCTION

Malaria, a cyclic febrile illness endemic in the tropics and subtropics, is the most important parasitic disease of humans. Five hundred million new infections occur annually, and close to 1% of those infected die. (Nussenzweig and Zavala, 1997) The disease is caused by one of four species belonging to the genus *Plasmodium; Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale* or *Plasmodium malariae.*

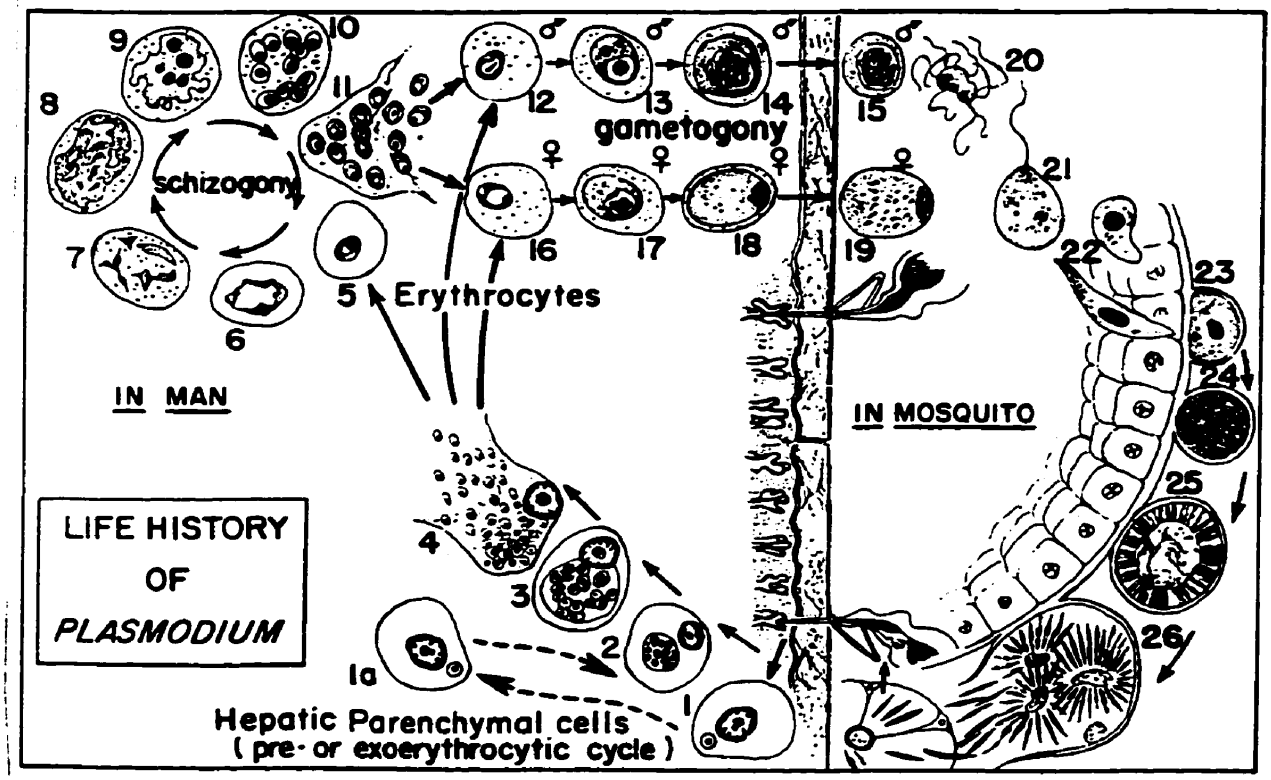


FIGURE 1 - LIFE CYCLE OF *PLASMODIUM*
1-4; Liver stage schizogony. 5-11; Erythrocytic schizogony. 12-19; Gametogenesis. 20-26; Sexual replication. Distinct life cycle stages: 4&11; Merozoite. 5-7; Ring. 8; Trophozoite. 9-10; Schizont. 15; Microgamete. 19; Macrogamete. 22; Ookinete. 23-25; Oocyst. 26; Sporozoite. (adapted from Markell *et al.*, 1992)

The life cycle is similar for all four species (Figure1). It is biphasic, consisting of both sexual and asexual replication in the mosquito vector, (Figure 1, 20-26), and multiple rounds of asexual replication, known as schizogony, in the human host. Schizogony occurs primarily during the erythrocyte stage, where invading merozoites pass through ring, trophozoite and schizont forms, altering the red cell as they grow (Luse and Miller, 1971) (Figure 1, 5-11). *P. falciparum*, the most virulent agent of human malaria, is unique in that the predominant form of parasite found in the peripheral circulation is the immature ring stage (Bignami and Bastianelli, 1889). The more mature trophozoite and schizont forms adhere to endothelial cells in microvascular capillary beds in a variety of organs including the brain, heart, lungs and intestine; a phenomenon known as sequestration (Miller, 1969). It has been proposed that sequestration is a parasite strategy to avoid splenic elimination of infected erythrocytes (IEs) (Howard and Gilladoga, 1989). This allows the parasite to complete its life cycle successfully in an area of low oxygen tension, and thus promotes effective multiplication. Consequently, cytoadherence is a major virulence factor and is central to the pathophysiology of cerebral malaria.

CEREBRAL MALARIA

Cerebral malaria is the most severe complication of *P. falciparum* infection. It primarily occurs in non-immune individuals i.e. inhabitants of non-endemic regions, young children from endemic areas, and residents of areas with unstable transmission. The condition carries a 20-50% fatality rate, although no residual neurological deficit occurs in the majority of patients who recover (Miller *et al.*, 1994).

Although clinical findings have led to various hypotheses, the pathogenesis of cerebral malaria remains incompletely understood. The most prevalent hypothesis is that IEs, sequestered in the microvasculature of the brain, mechanically obstruct blood flow leading to tissue hypoxia and coma, similar to cerebrovascular disease. Indeed, there is a correlation between parasitemia, cerebral capillary packing and the clinical syndrome of cerebral malaria (MacPherson *et al.*, 1985; Pongponratn *et al.*, 1991; Turner *et al.*, 1994). However, unexplained by this hypothesis is the lack of long-term neurological deficits in patients who recover from cerebral malaria, in contrast with the permanent cerebral damage which occures in stroke patients.

An alternative explanation of cerebral malaria pathogenesis centres on the involvement of nitric oxide (NO) (Clark *et al.*, 1991). In this model, elevated TNFα and IL-1 levels in patients with cerebral malaria (Kwiatkowski *et al.*, 1990), result in the release of NO by endothelial cells and smooth muscle. NO crosses the blood-brain barrier contacts neurons, interferes with normal signaling pathways and contributes to a reversible anesthetic state. Although this model is compatible with other clinical findings and a full recovery in survivors, it lacks solid experimental evidence. Other suggested theories of cerebral malaria pathogenesis include the release of toxic factors from IEs (Kwiatkowski *et al.*, 1997), as well as immune-complex deposition in vascular beds (Aikawa *et al.*, 1990).

Central to all theories, and a consistent pathological finding, is the presence of sequestered IEs in the brain (MacPherson *et al.*, 1985; Pongponratn *et al.*, 1991; Turner *et al.*, 1994). Thus, paramount to the understanding of cerebral malaria will be an elucidation of the molecular interactions involved in cytoadherence. Toward this goal, several endothelial cell

receptors and their corresponding IE ligands have been identified, however their respective roles in the pathogenesis of cerebral malaria have not been fully defined.

ENDOTHELIAL CELL RECEPTORS

Endothelial cells form the simple squamous epithelium which lines all blood vessels (Wheater *et al.*, 1993). Many membrane proteins and glycoconjugates are constitutively or inducibly presented on the luminal surface of these cells, often in an organ specific pattern. One of the primary functions of these molecules is to mediate intercellular adherence of circulating immune cells (Kuby, 1994). Several of these molecules have also been identified as receptors for IEs including clusters of differentiation molecule 36 (CD36) (Barnwell *et al.*, 1989), intercellular adhesion molecule 1 (ICAM-1) (Berendt *et al.*, 1989), thrombospondin (TSP) (Roberts *et al.*, 1985), endothelial leukocyte adhesion molecule 1 (ELAM-1), vascular cell adhesion molecule (VCAM) (Ockenhouse *et al.*, 1992), Chondroitin Sulfate A (CSA) (Rogerson *et al.*, 1995), clusters of differentiation molecule 31 (CD31) (Treutiger *et al.*, 1998), and P-selectin (Ho et al, 1998).

CD36

CD36 is a cell surface glycoprotein expressed on monocytes, erythroid precursors, mammary epithelial cells, and vascular endothelial cells in select tissues (Greenwalt and Mather, 1985; Knowles *et al.*, 1984). It was first identified as a platelet glycoprotein, GPIV, possessing remarkable resistance to proteolysis (Okumura and Jamieson, 1976; Podolsak, 1977). Many roles have been ascribed to it including various adhesion and transduction phenomena.

The estimated molecular weight of CD36 is 88 kDa when isolated from human platelets, although this weight varies depending on the tissue or cell type of origin, presumably due to cell type specific glycosylation (Okumura and Jamieson, 1976; Tandon *et al.*, 1989). Both O- and N- glycosidic linkages have been demonstrated, and the latter are believed to be responsible for the resistance of membrane-bound CD36 to proteolysis (Tandon *et al.*, 1989). It is predicted that the CD36 cDNA, isolated from a human placenta cDNA library, encodes a single chain protein with an open reading frame of 472 amino acids (Oquendo *et al.*, 1989). Sequence analysis reveals 10 cysteine residues, 4 at the extreme N- and C- termini (positions 3, 7, 464, 466) and 6 in an extracellular cysteine rich domain (residues 243 to 333). Other proteins related to CD36 include the lipoprotein scavenger receptors SR-BI/CLaI, and the lysosomal integral membrane protein LIMPII (Acton *et al.*, 1994). These proteins are also predicted to have a single chain topology, with at least 5 of the 6 cysteine residues conserved in the extracellular region. Recent studies demonstrate that all four terminal cysteines are palmitoylated, suggesting that CD36 spans the membrane at both ends (Tao *et al.*, 1996). As well, within the cysteine rich domain there are three disulfide bonds (242-310, 271-332, 312-321) (Rasmussen *et al.*, 1998). Other studies have suggested that a threonine residue at position 92 is differentially phosphorylated resulting in differential ligand binding, and that this molecule may dimerize in the membrane in response to various stimuli (Asch *et al.*, 1993).

When immunoprecipitated from platelets and other cell types, CD36 has been shown to be physically associated with *src*-related kinases, suggesting that it plays a role in signal transduction (Huang *et al.*, 1991). The activation of platelets and the induction of an oxidative burst in monocytes by CD36 ligands or anti-CD36 antibodies support this function

(Tandon *et al.*, 1989; Okenhouse *et al.*, 1988). Further, CD36 has been shown to participate in the phagocytosis of senescent neutrophils by macrophages (Savill *et al.*, 1991).

CD36 has been demonstrated to have a central role in many adhesive phenomena. It has clearly been shown to act as a platelet receptor for collagen, and a membrane receptor for TSP, (Tandon *et al.*, 1989; Asch *et al.*, 1987) and CD36 also binds lipoprotein of various densities (Calvo *et al.*, 1998).

CD36 also participates in the adherence of *P. falciparum in vivo*. Historically, the role of CD36 in cytoadherence was inferred from its coincident presence on various cell types that support IE cytoadherence, including monocytes, endothelial cells, platelets and C32 melanoma cells (Barnwell *et al.*, 1985). Studies using the anti-CD36 monoclonal antibody OKM5 confirmed the role of this protein as a receptor for IEs (Barnwell *et al.*, 1985; Ockenhouse and Chulay, 1988; Oquendo *et al.*, 1989). Subsequently, purified CD36 was shown to inhibit cytoadherence, and both purified as well as COS cell expressed protein were demonstrated to directly support IE binding (Barnwell *et al.*, 1985; Ockenhouse *et al.*, 1989; Oquendo *et al.*, 1989).

The molecular mechanisms of CD36-IE interaction are still not clearly understood. Early studies assumed that the OKM5 and IE binding sites are coincident since the antibody blocks cytoadherence. Thus when a portion of the OKM5 epitope was mapped to residues 155-183 by chimaeric protein expression and incomplete homologue-replacement mutagenesis, it was suggested that this region was also responsible for IE binding (Daviet *et al.*, 1995). However, recent data from our lab suggest that the CD36 epitope involved in IE binding is discontinuous and/or conformational (Crandall *et al.*, 1999). Cysteine scanning mutagenesis in which cysteines at positions 243, 272, 311, 313 and 322 were individually

replaced completely ablated or severely inhibited cytoadherence (Hull *et al.* Manuscript in Preparation). This strongly implicates this region in IE binding and suggests that the tertiary structure provided by disulfide bonds between these residues maintains the necessary IE binding conformation. In addition, a histidine at position 242 has been shown to be involved in the pH dependent binding displayed by IEs, further supporting the role of this region in IE binding (Serghides *et al.*, 1998; Marsh *et al.*, 1988).

INTERCELLULAR ADHESION MOLECULE 1 (ICAM1)

ICAM1 has also been identified as a receptor for IEs. ICAM1 is an 80 to 115 KDa membrane glycoprotein belonging to the immunoglobulin (Ig) superfamily. Structurally it is composed of five tandemly linked Ig domains, and is surface expressed as a monomer on lymphocytes, macrophages and vascular endothelium. Its primary physiological role is the cellular adhesion of immune cells via the integrin ligands (Kuby, 1994).

ICAM1 was first identified as a receptor for IEs using a COS-7 cell transfection system (Berendt *et al.*, 1989), and has since been used in purified form for *in vitro* studies (Ockenhouse *et al.*, 1992). The same authors identified the IE binding site which is located in the N terminal Ig domain at residues 14-22. Domain 2, although not directly involved, is conformationally linked to the binding site and is therefore also required in IE binding. Based on this knowledge, recombinant soluble ICAM-1, Ig domains 1 to 4, has been used to try and disrupt IE cytoadherence. Although this strategy was originally reported to be successful, (Ockenhouse *et al.*, 1992), subsequent studies found that sICAM1 cannot inhibit cytoadherence, and pointed to a rapid K_{off} rate as creating artifacts in the first study (Craig *et al.*, 1997).

OTHER RECEPTORS

Other IE receptors have also been identified by *in vitro* cytoadherence studies including thrombospondin (TSP), Chondroitin sulfate A (CSA), P-selectin, endothelial leukocyte adhesion molecule 1 (ELAM-1) and vascular cell adhesion molecule 1 (VCAM-1) and CD31.

TSP is an adhesive glycoprotein secreted by various cell types including certain endothelial cells and activated platelets. *In vitro*, it has been shown to bind IEs directly in purified form, and in cell bound form on C32 melanoma cells (Roberts *et al.*, 1985). However, not all cells that secrete TSP bind IEs, suggesting the requirement of a coreceptor. CD36 has been implicated as a cell surface receptor for TSP, and it has been suggested that in some instances TSP may act as a bridge between the IE and CD36, directly mediating cytoadherence, or inducing the dimerization of CD36 molecules which may lead to more effective IE binding, or intracellular signaling events (Daviet *et al.*, 1997; Asch *et al.*, 1987).

Another cell surface receptor for IEs is CSA. This molecule was first identified as mediating adherence to CHO cells and contributing to adherence to C32 melanoma cells *in vitro* (Rogerson *et al.*, 1995). Both lab-adapted strains and wild isolates were able to use this receptor suggesting that it possesses some clinical significance. Further studies revealed the involvement of this receptor in maternal malaria due to the extensive expression of CSA on the placenta (Fried and Duffy, 1996).

P-selectin, an endothelial molecule involved in neutrophil recruitment has also been found to bind IE *in vitro*. Similar to neutrophil adherence, IE binding to this molecule requires Ca^{2+} modulation of the lectin domain conformation prior to binding. However, a

monoclonal antibody directed against the neutrophil binding site of P-selectin did not inhibit IE binding suggesting that a distinct epitope may be involved in IE adherence.

Other cellular adhesion molecules, VCAM-1 and ELAM-1(Okenhouse *et al.*, 1992) and CD31 (Treutiger *et al.*, 1998), have also been identified as IE receptors *in vitro*. Binding to these receptors was stimulated in each instance by serial panning experiments with lab adapted parasites. Since then, the relevance of these molecules as *in vivo* receptors has been disputed (Udomsangpetch *et al.*, 1996; Turner *et al.*, 1994).

INFECTED ERYTHROCYTE LIGANDS

PfEMP1

In malaria endemic settings individuals are infected several times with *Plasmodium falciparum* before protective, non-sterile, immunity is acquired. It has long been recognized that geographically distinct strains of *P. falciparum* are immunologically distinct (Jeffery, 1966; Hommel *et al.*, 1983). *In situ* studies of Gambian children have demonstrated that in naïve individuals, convalescent serum specifically recognizes homologous parasites but rarely heterologous organisms (Marsh and Howard, 1986). This verifies the existence of variant epitopes on parasites from a single geographic location, and suggests the existence of immunologically distinct strains within a single parasite population.

A study conducted by Leech and colleagues confirmed the existence of a group of trypsin-sensitive, high molecular weight proteins from geographically distinct strains, PfEMP1 (Leech *et al.*, 1984), and subsequent *in vitro* studies demonstrated that this family of proteins exists within a single clone (Biggs *et al.*, 1991). Subcloning experiments indicate that these proteins demonstrate a switch rate of approximately 2.4% per generation (Roberts

et al., 1992). Antigenic variation is an evasion mechanism used by other microorganisms such as *Trypanosoma brucei*, and *Haemophilus influenzae* to evade the host immune response. It was therefore proposed that the delay in onset of clinical immunity to *P. falciparum* infection could be attributable to antigenic variation of the PfEMP1 molecues and that in an endemic setting individuals do not develop immunity until they have been exposed to all or most of the different antigenic variants of the local parasite population (Marsh and Howard, 1986).

Several indirect pieces of evidence support the role of PfEMP1 as a cytoadherence ligand. The first is that convalescent sera, presumed to recognize PfEMP1, inhibit cytoadherence in a strain specific manner (Udeinya *et al.*, 1983). In addition, changes in antigen phenotype *in vitro*, are frequently accompanied by changes in cytoadherent phenotypes (Roberts *et al.*, 1992; Biggs *et al.*, 1992; Biggs *et al.*, 1991). The further demonstration that PfEMP1 molecules change with cytoadherence phenotype also suggests that this molecule is involved in both cytoadherence and antigenic variation (Biggs *et al.*, 1992).

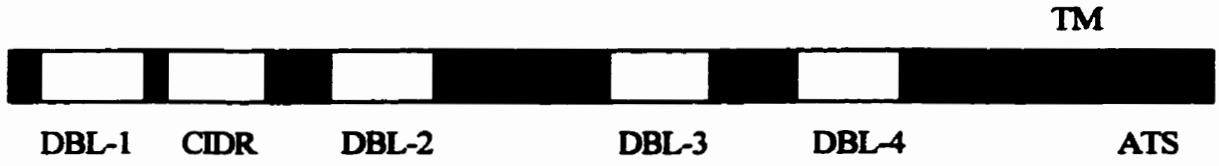
More recent evidence demonstrates that PfEMP1 can interact directly with CD36, ICAM1 and TSP, and that a peptide fragment based on predicted residues 1-179 from the *var-1* gene encoding PfEMP1 of the Malayan Camp clone (*MCvar-1*) reduces IE binding to CD36 (Baruch *et al.*, 1996; Baruch *et al.*, 1997). These two studies demonstrate directly that PfEMP1 is involved in cytoadherence.

VAR GENE FAMILY

PfEMP1 is encoded by the highly diverse family of genes designated *var* (Su *et al.*, 1995; Smith *et al.*, 1995; Baruch *et al.*, 1995). These genes are typically 7 to 10 kb and are encoded in a two exon format. The large first exon, 5 to 8 kb, represents the extracellular and transmembrane domains. Sequence analysis of the *var* genes examined to date reveal that this exon is highly polymorphic, a result in accordance with the role of PfEMP1 in antigenic variation. The conserved amino acids are concentrated in small segments and form structural motifs present in all *var* genes (Figure 2).

Most notable are a variable number of cysteine-rich extracellular Duffy Binding Like domains (DBLs); ~200 residue segments that share identity with erythrocyte binding proteins EBA-175 of *P. falciparum* and the Duffy binding antigens of *P. vivax* and *P. knowlesi*. All *var* genes contain between two and four of these domains, each with its own amino acid signatures. The DBL1 motif is consistently the closest to the 5' end of the *var* gene, and its signatures are the most conserved. The other DBLs, which have less discriminating signatures, may be present in shuffled arrangements, or absent all together. Another conserved element is the cysteine rich interdomain region (CIDR). The amino end of this 300-400 residue motif shares some homology with DBLs; it is located between DBL1 and the subsequent DBL domain. Downstream of the last DBL is a polymorphic segment of variable length, followed by a hydrophobic region, presumed to encode the transmembrane domain. A ~1kb intron which follows the transmembrane domain separates the two exons (Su *et al.*, 1995; Smith *et al.*, 1995; Hernandez-Rivas *et al.*, 1997). The second exon encodes a relatively conserved, acidic region which is believed to function as a cytoplasmic tail (Acid Tail Sequence, ATS).

A)



B)

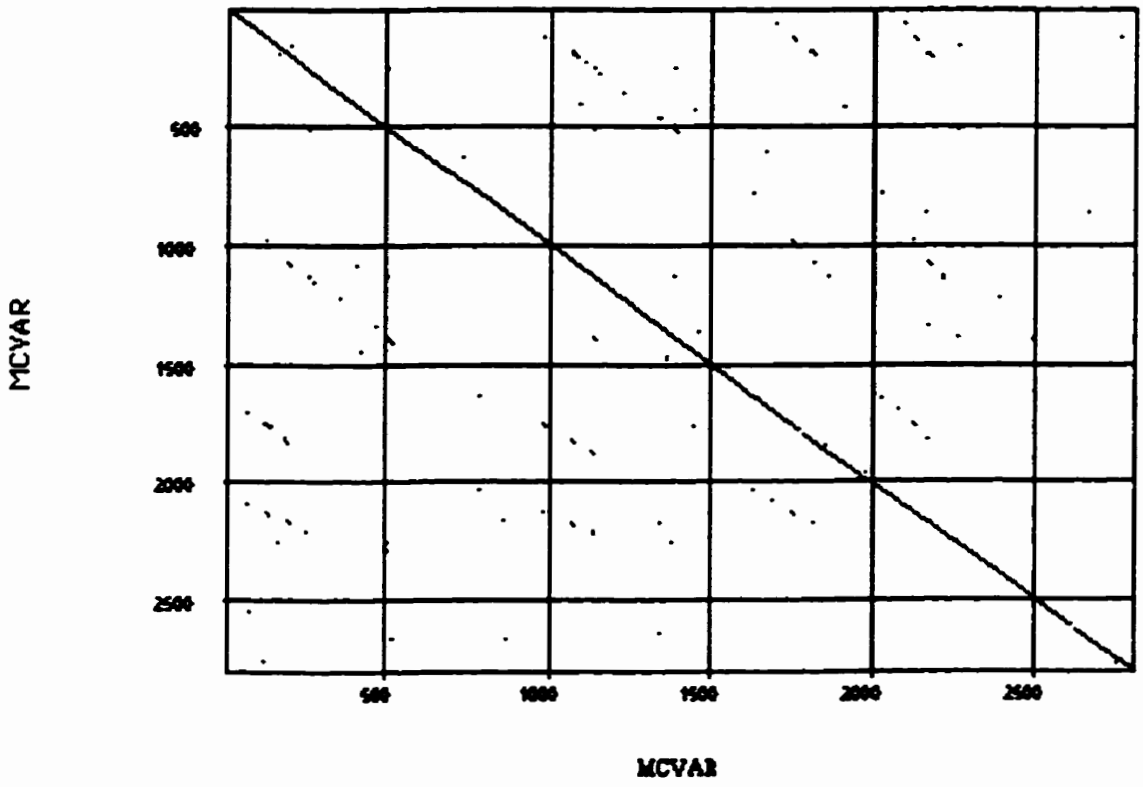


FIGURE 2 - STRUCTURAL COMPONENTS AND PROTEIN MATRIX ANALYSIS OF THE *MCVAR1* GENE
A) Schematic representation of *var* genes demonstrating Duffy Binding Like Domains DBLs (in white), the Cysteine rich interdomain (CIDR), the transmembrane domain (TM), the acid tail sequence (ATS) and areas of variable sequence (grey) (Adapted from Su *et al.*, 1995).
B) The MCvar-1 gene was compared to itself using a window size of 10 and a minimum match score of 60%. Areas of sequence repeat (i.e. DBLs) are clearly seen parallel to the diagonal in four areas.

If the predicted protein sequence of the Malayan Camp *var* gene 1 (*MCvar-1)* gene is compared against itself in a protein matrix, the location of four DBL domains can be visualized as lines parallel to the central diagonal. (Figure 2B)

It was demonstrated using a probe complementary to the ATS that parasites of many strains possess an estimated 50 to 150 *var* genes per organism (Su *et al.*, 1995). Assuming that each *var* gene occupies12 kb of genomic DNA, up to 6% of the genome may be involved in the coding of this family. *var* genes are present on most chromosomes, and may exist individually, or be arranged in tandem arrays (Su *et al.*, 1995; Hernandez-Rivas *et al.*, 1997). Two dimensional pulse field gel electrophoresis (PFGE) and Northern blot analysis have revealed that members of this family are present and transcribed from many chromosomes both from internal and subtelomeric locations (Su *et al.*, 1995; Rubio *et al.*, 1996; Hernandez-Rivas *et al.*, 1997). The subtelomeric location of *var* genes is significant since these regions are highly recombinogenic, (Kemp *et al.*, 1985), and the existence of *var* genes at these locations may contribute to the extreme diversity of this family through the recombinational generation of novel variant forms. Indeed, there is some evidence that *var* genes located at the ends of heterologous chromosomes are more closely related to each other than they are to internal *var* genes of either homologous and heterologous chromosomes (Rubio *et al.*, 1996).

Little is known about the regulation of transcription of the *var* gene family. Within a culture, multiple *var* genes may be expressed simultaneously, although some are transcribed only at low levels (Smith *et al.*, 1995; Hernandez-Rivas *et al.*, 1997). It was proposed that, as with African trypanosome VSG genes, *var* gene expression may be controlled by duplicative transposition of silent genes into active transcription sites. It was found that large scale

genomic rearrangements required by such a model do occur, but they do not correlate with *var* gene expression (Su *et al.*, 1995; Smith *et al.*, 1995). This coupled with the existence of both subtelomeric and internal expression sites seems to refute this model.

The highly polymorphic nature of Exon 1 is consistent with the role of PfEMP1 in antigenic variation. RT-PCR studies have shown a highly significant correlation between expressed *var* genes and antigenic phenotype (Smith *et al.*, 1995). There is also some evidence linking cytoadherence and *var* gene expression. For example, antibodies raised against recombinant *MCvar-1* gene product inhibited cytoadherence to CD36 and recognized an antigen in the electron dense knob structures of infected cells (Baruch *et al.*, 1995). To reconcile the extreme variability of the extracellular domain encoded by *var* genes with the consistent nature of cytoadherence, it has been proposed that the two 5' elements, DBL1 and CIDR, form a conserved head structure that serves as a functional ligand (Su *et al.*, 1995). A distinct *var* gene of clone R29 has also been associated with rosetting, again implicating *var* gene products as parasite derived ligands (Rowe *et al.*, 1997).

BAND 3

Band 3 is the major anion transporter of human erythrocytes. Present in approximately one million copies per cell, it exchanges Cl^-/HCO_3^- and thus plays a central role in the transport of CO_2. It was first associated with *P. falciparum* infection when truncated versions of the protein were identified in IEs (Winograd *et al.*, 1987; Winograd and Sherman, 1989). This phenomenon may be similar to the apparent degradation of band 3 in senescent erythrocytes or red blood cells from individuals suffering from various hemolytic anemias (Kay *et al.*, 1990). Subsequently, mouse monoclonal antibodies generated against

intact IE were found to recognize altered band 3 molecules in infected but not uninfected erythrocytes (Crandall and Sherman, 1991a). The isolates used in these studies were from diverse geographic origins thus demonstrating that this proteolytic phenomenon is not strain specific. Although the protease responsible remains unknown, it is presumed to be of parasite origin since one strain, Honduras CDC-1, produced a consistently larger band 3 degradation product.

The link between band 3 neoantigens and cytoadherence was suggested when the anti-band 3 monoclonal antibodies were found to inhibit IE cytoadherence to C32 melanoma cells and CHO-CD36 cells *in vitro* (Crandall and Sherman, 1994a; Guthrie *et al.*, 1995). Subsequent studies examining the putative exofacial loops of human band 3 recognized by the mouse monoclonal antibodies identified two regions whose peptide sequences inhibited cytoadherence *in vitro*; residues 547-553 and 824-829. *In vivo* these peptides also appeared to interfere with cytoadherence, leading to the liberation of mature forms of IE into the general circulation of *Aotus* and *Saimiri* monkeys (Crandall *et al.*, 1993). This peptide pair, which has been named *Pfalhesin,* has been implicated as a ligand in rosetting, the alternative adhesive phenotype of IE (Crandall and Sherman, 1994a; Crandall *et al.*, 1994b).

In addition, clinical studies have revealed that the sera from immune adults living in malaria-endemic areas recognize band 3 neoantigens, but that sera from non-immune adults living in non-endemic areas do not (Crandall *et al.*, 1995). This indirect evidence supports the laboratory findings mentioned above and draws a link to human *P. falciparum* infection. As well, the production of autoantibodies against specific regions of band 3 in the endemic setting lends support to the proposed mechanism of action of band 3 in cytoadherence i.e.

proteolytic degradation of the band 3 changes the surface topography of the molecule, exposing previously cryptic adhesive elements that are able to interact with CD36.

OTHER LIGANDS

The existence of additional IE ligands has also been proposed. For example Sequestrin is a parasite-derived, trypsin-sensitive, 270 kDa protein found on the surface of IE (Ockenhouse *et al.*, 1991). It was first identified using anti-idiotype polyclonal antibody raised against the cytoadherence inhibiting monoclonal antibody OKM8. Affinity purification studies have shown that it interacts directly with CD36-sepharose, and IFA studies have confirmed its presence on a wide range of parasite lines and wild isolates (Ockenhouse *et al.*, 1991).

Two trypsin resistant molecules presumed to mediate adherence to unidentified receptors on C32 melanoma cells and human umbilical vein endothelial cells (HUVECs) have also been identified (Chaiyaroj *et al.*, 1994a; Chaiyaroj et al, 1994b). Since these molecules have a molecular weight similar to PfEMP1 and share other biochemical characteristics, it has been suggested that they may represent variants of PfEMP1. However, other features such as detergent solubility, temporal expression and host vs. parasite origin have not yet been examined, so such a classification is premature.

In addition, the sub-telomeric region of parasite chromosome 9 has been implicated in cytoadherence (Chaiyaroj et al, 1994a; Day *et al.*, 1993). *In vitro*, many parasite lines undergo a 0.3 Mb deletion in this region, facilitated by a break point in the Break Point Open Reading Frame (Bourke *et al.*, 1996; Day *et al.*, 1993). The deletion is accompanied by a coincidental loss of cytoadherence, indicating the importance of some element in this region.

At first it was suggested that a parasite ligand was encoded here, but with the discovery of many new ligand-receptor combinations, and the genome-wide distribution of the *var* genes, it is more likely that this region encodes some regulatory gene involved in ligand expression or protein trafficking.

KNOBS

The ultrastructure of erythrocytes infected with *P. falciparum* is altered during the course of the asexual life cycle (Trager *et al.*, 1966). As parasites mature into trophozoites and schizonts, electron dense protrusions known as knobs develop. Electron microscopy reveals that these knobs are contact points for attachment to endothelium and thus they are proposed to play an important role in cytoadherence (Luse and Miller, 1971). *In vivo,* almost all IE are of the knobby phenotype.

In vitro, some parasite lines lose their ability to produce the knobby phenotype (K^+), becoming knobless (K^-). This mutation is associated with a chromosome 2 rearrangement and truncation of the gene encoding Knob Associated Histidine Rich Protein (KAHRP), a major structural component of knobs (Pologe and Ravtech, 1986; Culvenor *et al.*, 1987; Pologe *et al.*, 1987). Despite their altered phenotype these parasites may retain their ability to bind CD36 in purified or cell bound form, in static adhesion assays (Biggs *et al.*, 1990). However, when studied under flow conditions *in vivo* or *in vitro*, K^- parasites do not cytoadhere, again pointing to the importance of knobs in cytoadherence (Langreth and Peterson, 1985; Crabb *et al.*, 1997). Interestingly, some K^+ lines become non-adherent, indicating that the knob structure alone is not sufficient for binding.

Further support for the involvement of knobs in cytoadherence is immunoelectron microscope studies localizing parasite ligands to these structures. PfEMP1 was directly demonstrated to be associated with >50% of knob structures on the exterior IE surface (Baruch *et al.*, 1995). Band 3 neoantigens were inferred to be associated with knobs when human Ig from non-immune individuals reacted specifically with these structures (Winograd and Sherman, 1989). It was postulated that anti-band 3 autoantibodies involved in senescent cell antigen recognition were responsible for this staining phenomenon. However, recent data indicate that sera from non-immune individuals do not recognize peptides modeled on band 3 neoantigens, thus casting some doubt on this theory (Crandall *et al.*, 1995).

INTEGRATIVE STUDIES

In spite of the progress made in identifying endothelial cell receptors and IE ligands *in vitro*, the relative contributions of these molecules *in vivo* remains unclear. For the most part, the various proposed receptors or ligands have been considered in isolation, and few comprehensive models of cytoadherence have been proposed. However some methodologies, such as shear stress and wild isolate binding studies as well as post-mortem microscope evaluations, have allowed partial integration of the many potential receptor/ligand interactions into models of cytoadherence, and emphasized the importance of this phenomenon in disease pathogenesis.

WILD ISOLATE BINDING ASSAYS

Typically, the biological significance of endothelial cell receptors has been examined by assessing the *in vitro* binding phenotype of peripherally drawn wild isolates. It has been

repeatedly demonstrated that virtually all *P. falciparum* wild isolates consistently bind in high numbers to CD36, suggesting that it is a major *in vivo* receptor (Ockenhouse *et al.*, 1991; Udomsangpetch *et al.*, 1996). However, the exact role of this receptor in cerebral malaria is still unclear since wild isolates from patients with complicated and uncomplicated malaria alike, bind CD36 equally well (Ockenhouse *et al.*, 1991). Binding to other receptors including ICAM1, VCAM, ELAM, TSP, and P-selectin is much more variable, and quantitatively inferior to CD36 binding. Additionally there is no clear correlation between cerebral malaria and any of these receptors, making their true biological significance harder to discern (Ockenhouse *et al.*, 1991; Udomsangpetch *et al.*, 1996).

One limitation to this type of study is the small sample size, since significant trends in binding phenotype might be missed. To overcome this problem, a larger study examining 150 samples was recently conducted (Newbold *et al.*, 1997). Again, binding to CD36 at quantitatively high levels was found to be consistent regardless of clinical severity, and binding to ICAM1 and VCAM quantitatively inferior. However, binding to ICAM1 was found to be significantly correlated with cerebral malaria isolates, suggesting the importance of this receptor in disease pathogenesis.

A potential limitation of studies relying on wild isolates is the peripheral nature of the drawn blood. Since only ring stage parasites circulate in *P. falciparum* infection, this necessitates the *in vitro* culture of samples for 12 to 36 hours. This *ex vivo* manipulation might affect binding phenotype either by environmental signals such as altered O_2 and CO_2 levels, or removal of stimulating cytokine factors. Additionally, it has been shown that peripherally drawn cultures do not reflect the binding phenotype of sequestered parasites binding to CSA in the placenta (Fried and Duffy, 1996). This suggests that the binding

phenotype of parasites sequestered in the brain or other organs might not be accurately reflected in the samples used in these studies. Consequently, conjectures of disease pathogenesis *in vivo* based on these results must be made with caution.

An additional limitation of *in vitro* wild isolate binding assays is that they are performed under static rather than flow conditions, which exist in the human body. To correct this shortcoming, *ex vivo* flow systems have been developed (Cooke *et al.*, 1994; Udomsangpetch *et al.*, 1997; Siano *et al.*, 1997). At physiologic shear stress, *P. falciparum* isolates, wild or lab adapted, roll on ICAM-1, VCAM-1 and P-selectin, and statically adhere to CD36, CSA and TSP (Cooke *et al.*, 1994; Cooke *et al.*, 1996; Udomsangpetch *et al.*, 1997; Ho *et al.*, 1998). This suggests a slow K_{off} rate for CD36 and a rapid K_{off} rate for the other receptors. In general, the binding phenotype under these conditions follows the same pattern as static adherence assays i.e. CD36>ICAM-1>other receptors. Despite the very low level of IE rolling on TSP (Cooke *et al.*, 1994) the addition of soluble TSP has been reported to significantly increases IE binding to CD36, again suggesting some bridging role of TSP in cytoadherence (Rock *et al.*, 1988; Siano *et al.*, 1997).

POST-MORTEM STUDIES

Post-mortem studies examining receptor expression and parasite packing have also been used to draw conclusions about the biological significance of various receptors and support or exclude their role in the pathogenesis of cerebral malaria. For example, CD36, although expressed constitutively at high levels in muscle, lung and liver microvaculature, is expressed at very low levels in the brain of those suffering from cerebral malaria, uncomplicated malaria, or no disease (Turner *et al.*, 1994; Barnwell *et al.*, 1989). Despite

this low level, there is a significant association between CD36 expression and the pattern of cerebral sequestration ($p<0.001$) (Turner *et al.*, 1994). In contrast, ICAM-1 and E-selectin are upregulated through out the body of infected individuals, and both are strongly correlated with parasite sequestration in cerebral vessels ($p<0.0001$) (Turner *et al.*, 1994). Upregulation of the expression of ICAM-1 and E-selectin, but not CD36 in infected individuals is thought to be brought about by cytokine modulation. Indeed, individuals suffering from cerebral malaria have significantly elevated TNFα levels, (Kwiatkowski *et al.*, 1990; Grau *et al.*, 1989) suggesting a link between receptor upregulation and disease pathogenesis.

Once again, there are certain limitations of these studies which restrict their usefulness in the elucidation of *in vivo* disease pathogenesis. The most obvious is the post-mortem nature of the samples. Since many individuals are in coma for hours or days before death, the snapshots captured by these studies may be markedly varied. Additionally several hours may elapse between death and post-mortem so that the samples collected may not reflect the vasculature at the time of death. As well, the fixing processes may introduce artifact, or destroy receptors, so that accurate endothelial cell receptor and IE adherence distributions may be difficult to determine.

CYTOADHERENCE MODELS

The most comprehensive current model of cytoadherence likens this process to leukocyte recruitment in inflammatory processes (Cooke *et al.*, 1994; Udomsangpetch *et al.*, 1997). In this model, cytoadherence is viewed as a mutli-step process in which IEs tether, roll and statically adhere by interacting with various endothelial cell receptors. Although IEs can adhere directly to CD36 and CSA during flow, it is postulated that this occurs only in

peripheral organs such as the liver, lungs, placenta and muscle. In cerebral vasculature where the levels of expressed CD36 are very low (Turner *et al.*, 1994), rolling on other endothelial cell receptors is hypothesized to slow the IEs sufficiently to facilitate static adherence to CD36 (Cooke *et al.*, 1994).

It was alternatively suggested that the combined cellular attachment to multiple endothelial receptors that support rolling *in vitro* might mediate static adherence *in vivo* thus contributing to occlusive pathology in the brain (Udomsangpetch *et al.*, 1997). The induced upregulation of ICAM-1 and other receptor molecules in the cerebral microvasculature and the elevated levels of circulating TNFα levels in individuals suffering from cerebral malaria support this hypothesis (Turner *et al.*, 1994; Kwiatkowski *et al.*, 1990).

Since leukocyte recruitment involves many receptor/ligand interactions, this model implicitly suggests the involvement of multiple IE ligands. However, for the most part IE ligand involvement in adherence to multiple receptors has been neglected. The only model proposed that addresses this issue suggests that the conformational arrangement and variable presence of DBL and CIDR domains of PfEMP1 dictates binding to CD36, ICAM-1 and TSP alike (Su *et al.*, 1995). Although direct interaction of PfEMP1 with each of these receptors supports this hypothesis, the model fails to account for the incomplete inhibition of binding to CD36 caused by anti-PfEMP1 antibodies, or the potential contribution of other IE ligands to binding to these and other receptor molecules.

These preliminary models amply demonstrate the need to consider the involvement and potential interaction or cooperativity of the several endothelial cell receptors and IE ligands in any cytoadherence study, and point to the lack of such a comprehensive perspective in current research efforts.

RATIONALE FOR STUDIES

Cerebral malaria is the most severe and fatal complication of *P. falciparum* infection. Central to all models of the pathogenesis of cerebral malaria is the ability of the parasite to cytoadhere to brain endothelial cells, a process known as sequestration. Thus paramount to the understanding of cerebral malaria is the elucidation of the molecular interactions involved in cytoadherence. To date, several endothelial cell receptors and IE ligands have been identified, although their respective roles in cytoadherence and cerebral malaria remain for the most part unclear. Detailed time course studies could help define the roles of a subset of these adhesive molecules and provide some insight into their importance in sequestration.

HYPOTHESIS

We hypothesize that a receptor that plays a pivotal role in sequestration should support *in vitro* binding of mature IEs at high levels for the entire duration of their presence in culture. Additionally, any ligand contributing to cytoadherence should be present on the IE surface at the time of cytoadherence competence. For molecules of parasite origin, this would require transcriptional or post-transcriptional control facilitating stage specific production.

SPECIFIC OBJECTIVES

1. To determine the temporal onset of IE binding to CD36 and ICAM1.
2. To examine and correlate the surface presence of PfEMP1 and band 3 neoantigens with cytoadherence.
3. To explore transcriptional control of PfEMP1.

MATERIALS

Products and reagents described in the methods section below were obtained from the following sources. HEPES, sodium bicarbonate, Giemsa stain, sorbitol, gelatin (175 bloom), fetal bovine serum, Bis-Tris, Saponin, Tri-Reagent, and FITC were purchased from Sigma Aldrich Canada. Gentamicin, geneticin, RPMI medium 1640, trypsin EDTA, Taq DNA Polymerase, and AMV Reverse Transcriptase came from Gibco BRL. All tissue culture plastic-ware used was made by Corning. For RT-PCR, Promega RQ1 RNase-free DNase and RNase inhibitor RNasin™ were used and the random hexanucleotides were purchased from Pharmacia Biotechnologies. Silver Stain Express Kit and 10% TBE Polyacrylamide Gels were made by Novex. Qiaquick Gel Extraction Kit was purchased from Qiagen Canada. The TA Cloning Kit was from Invitrogen and the Sequenase PCR Product Sequencing Kit from United States Biochemicals (USB/Amersham). All secondary antibodies were purchased from Calbiochem. Primary antibodies 1C4, 3H3 and 1F4 were gifts provided by Dr. Crandall (University of Toronto) and Dr. I.W. Sherman (University of California, Riverside), ATS contributed by Dr. J.C. Reeder (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) and MBP-r179 donated by Dr. D. Baruch (Laboratory of Parasitic Diseases, National Institutes of Health, Maryland, USA). All blood products were provided by local donors.

METHODS

PARASITE MAINTENANCE

Plasmodium falciparum parasites of the ItG clone were maintained *in vitro* at 37°C under reduced O_2 conditions (Trager and Jensen, 1976). Cultures were grown in washed human A+ erythrocytes at 5% hematocrit in HEPES/bicarbonate buffered RPMI 1640 medium supplemented with 10% heat inactivated human A+ serum and 25μg/ml Gentamicin (RPMI-A+). Isolates from donors were cultured identically, regardless of the donor patient's own blood type. All parasite cultures were monitored daily by microscopic examination of Giemsa stained thin films.

PARASITE SYNCHRONIZATION

Two techniques were used to maintain careful parasite synchrony: gelatin floatation and sorbitol lysis. Both of these methods depend on the rigid, expanded nature of the mature IE. In gelatin floatation, uninfected erythrocytes and non-deformed ring stage IE form rouleaux and sink through the gelatin solution. Since the mature IE are enlarged, they cannot participate in rouleaux formation and most remain suspended in the gelatin solution. In sorbitol lysis, the mature forms are more sensitive to the high osmolarity of the 5% sorbitol solution and burst rapidly, leaving only the ring form of the parasite in the culture. Both approaches have been used independently and in combination to create and maintain synchronous cultures (Lambros and Vanderberg, 1979; Paslov *et al.*, 1978; Vernot-Hernandez *et al.*, 1984). Gelatin floatation was performed by incubating 50% hematocrit culture with 0.25 volumes gelatin for 30 minutes to 1 hour at 37°C (Paslov *et al.*, 1978). Sorbitol lysis of mature IEs was achieved by incubation of packed culture erythrocytes in

5 volumes of a 5% sorbitol solution for 5 minutes at room temperature (Lambros and Vanderberg, 1979). To roughly synchronize cultures, trophozoites and schizonts of all ages were selected by gelatin floatation and subsequently incubated for 6 to 12 hours. During this time many of the parasites ruptured and reinvaded new erythrocytes, taking on the ring form. Remaining mature IE were eliminated by sorbitol lysis. For time course assays, this synchronization procedure was performed on parasite cultures during two consecutive schizogony cycles. Final synchronization was performed in a similar fashion with a stringent gelatin floatation of the culture when the majority of the parasites were in the late schizont stage, and a reduced, 4 hour, interim incubation for parasite rupture. The result was a culture of ring stage parasites less than 4 hours divergent in age.

BINDING ASSAYS

Chinese Hamster Ovary (CHO) cells stably transfected with genes coding for CD36, ICAM, or vector alone (CHO-CD36, CHO-ICAM and CHOϕ respectively), were maintained in *in vitro* culture (Hasler *et al.*, 1993). HEPES/bicarbonate buffered RPMI medium 1640 supplemented with 10% fetal bovine serum, 25μg/ml gentamicin and 40μg/ml geneticin was used for this purpose (RPMI-10). Cells were detached with 10% trypsin/90% EDTA, and seeded into 12 well plates or 35mm petri dishes at approximately 10% confluency (Crandall *et al.*, 1991b).

For time course binding studies, CHO-CD36 and CHOϕ cells were fixed with 4% formalin/PBS for 20 minutes and stored in Bis-Tris Saline (50mM Bis-Tris, 130mM NaCl, pH 6.8)(BTS) at 4°C until used. For CHO-ICAM cells, preliminary studies indicated that IE adherence was diminished by CHO cell fixation and decreasing chamber size. Therefore,

they were used unfixed and seeded in triplicate in 35 mm petri dishes at 12 hour intervals to prevent cell overgrowth. To verify equal ICAM expression at all time points, paired samples were assessed by immunofluoresence assay (IFA).

Parasite culture at 5% hematocrit and 2-8% parasitemia was added to adherent CHO cells and incubated for 1 hour at room temperature an on orbital shaker. Non-adherent erythrocytes were then removed by rinsing with BTS. Cytoadherence was assessed by examining unstained cells with a Nikon TMS-F inverted microscope and counting the number of IEs bound per CHO cell. Counting was performed immediately for live assays and within 12 hours for fixed assays.

PARASITE PANNING

Adherent CHO cells, originally seeded at 30% confluence in a 25cm^2 flask, were incubated for 1 hr at room temperature under reduced O_2 conditions with 3 ml parasite culture; 5% hematocrit, 2-8% parasitemia. The supernatant was aspirated, and a portion of the remaining non-adherent erythrocytes removed by a single rinse with unsupplemented, sterile, RPMI medium 1640. RPMI-A+ and human erythrocytes were then added to the CHO cells with adherent IEs and the cultures incubated overnight to allow IE rupture and re-invasion. Ring stage parasites, which are non-adherent, were removed the following day and cultured normally. To ensure the removal of all CHO cells, cultures were transferred to a fresh flask 2-4 hours following the initial rescue.

RNA Isolation

Culture samples were collected in one of two ways. For large scale RNA isolation, packed erythrocytes from 10 ml cultures were resuspended in 1.5 volumes 0.15% saponin, vortexed and then centrifuged 3 minutes at 500 g following the addition of 10 volumes unsupplemented RPMI medium 1640. The resultant pellet containing naked parasites was then resuspended in 1ml Tri-Reagent. Alternatively, the erythrocytes of 1.5ml of untreated culture were combined with 1ml of Tri-Reagent BD. 20 µl of 5N acetic acid were added to all samples prior to storage at -70°C.

RNA was extracted from samples according to the manufacturer's protocol, based on the method originally reported by Chomczynski and Sacchi, 1987. Briefly, 200 µl chloroform was added to thawed samples, vortexed briefly, and the phases separated by centrifugation at 12500 rpm, 4°C, for 15 minutes. The aqueous layer was then reextracted with 500 µl of Tri-Reagent or Tri-Reagent BD to remove remaining traces of contaminating DNA. RNA was precipitated with 0.9 volumes of isopropanol and centrifugation at 4°C. The RNA pellet was washed with ice cold 75% ethanol, air dried and resuspended in RNase-free water. RNA concentration and purity was then determined by optical density measurement at 260µm and 280µm.

Subsequently, any remaining DNA contamination was eliminated from each sample by a 15 minute incubation with 2 units of RQ1 RNase free DNase at 37°C in a permissive buffer containing 50 mM NaCl, 10 mM Tris-HCl pH7.9, 10 mM $MgCl_2$, 1 mM DTT supplemented with 40 units RNasin™. Following this treatment, the enzyme was deactivated by the addition of EDTA and heat treatment at 65°C for 15 minutes.

RT-PCR AND PRODUCT ANALYSIS

1μg total RNA was reverse transcribed using 0.2μg random hexanucleotide primers and AMV reverse transcriptase under recommended conditions (100 mM Tris-HCl pH 8.3, 10 mM $MgCl_2$, 10 mM DTT, 100 mM KCl, 500 μM each dATP, dCTP, dGTP, dTTP, 1hr at 37°C)

Amplification of the cDNA was then carried out with Taq DNA polymerase under the recommended conditions (20 mM Tris-HCl pH 8.4, 50 mM KCl, 0.2 mM each dATP, dCTP, dGTP, dTTP, 1.5 mM $MgCl_2$, 0.5 μM each primer and 1.25 U Taq). In addition to cDNA amplification, equal quantities untreated RNA extract were subjected to PCR as a control to test for the presence of DNA contamination.

Degenerate primers UNIEBP5' and UNIEBP3' (Peterson *et al.*, 1995)(Table 1) designed to amplify the DBL region of most *var* genes, were used to amplify a region of the DBL domains represented in the sample: 1 min at 95°C; 35 cycles of 1 min at 95°C, 1 min at 45°C, 1 min at 72°C; terminal extension of 7 min at 72°C. Primers ATSF/ATSR and ACTF/ACTR (Table 1) were used to amplify a segment of the conserved exon 2 of *var* genes and a portion of the *Pf-actin I* gene (Wesseling *et al.*, 1989), respectively. Cycling conditions were similar to those for the DBL primers except that the annealing temperature was 54°C, and the concentration of ATSF/R primers was increased to 1 μM.

PCR products were analyzed by electrophoresis through 2% agarose gels containing 0.3μg/ml ethidium bromide or through 5% polyacrylamide gels. Visualization was then achieved using UV light or by silver staining, respectively.

ATS and ACT PCR products were sequenced directly using the Sequenase PCR Product Sequencing Kit, according to the manufacturer's directions. DBL products were initially TA cloned, with subsequent amplification and sequencing of individual inserts.

TABLE 1 - PRIMER PAIR SEQUENCES

NAME	REGION RECOGNIZED	PRIMER SEQUENCE
UNIEBP5'	*var* DBL	5'-CC(A/G)AG(A/G)AG(A/G)CAA(G/A)AA(C/T)TATG-3'
UNIEBP3'	*var* DBL	5'-CCA(A/T)C(T/G)(T/G)A(A/G)(A/G)AATTG(A/T)GG-3'
ATSF	*var* Exon 2	5'-TAGTGGTATIGAIITAATIAAIGACGCAC-3'
ATSR	*var* Exon 2	5'-CCACTATGTGTCTCATTTTCCCAC-3'
ACTF	*Pf-actin I*	5'-CAGGAGATGATGCACCTCGTTC-3'
ACTR	*Pf-actin I*	5'-CATCTCCACTATCTAACACAATACC-3'

SSCP ANALYSIS OF *VAR* GENE AMPLICONS

PCR products were used directly, or the bands of interest were excised from agarose gels and eluted using QIAquick Gel Extraction Kit. 200 ng to 500 ng DNA were denatured with 1/10 volume 0.5M NaOH/10mM EDTA, 1/10 volume 5% bromophenol blue in formamide loading buffer and heat (42°C for 5 min). Samples were snap cooled on ice and rapidly loaded to maintain the ssDNA, and then run with 0.6X TBE running buffer (0.54 M Tris-borate, 1.2 mM EDTA) at constant voltage through 10% polyacrylamide gels. SSCP patterns were visualized by ethidium bromide staining (0.5μg/ml in TBE) or silver staining.

IMMUNOFLUORESENCE ASSAYS/CONFOCAL MICROSCOPY

Thin smears of parasite cultures were fixed for 10s in ice cold methanol and air dried. Sections of the slide were ringed with a Pap-pen defining a hydrophobic outline and then

dilutions were made in either RPMI-10 or 0.5% BSA in unsupplemented RPMI medium 1640. Fluorescence was assessed using an EFD-3 Nikon microscope with a DS-EPI-FI epifluorescent attachment or a Carl Zeiss LSM 410 inverted laserscan confocal microscope equipped with an Ar/Kr Chanichran Laser wavelengths 488/568/647. Antibody 1C4 was purified using Econopac Protein A cartridges and directly labeled with FITC by coupling at 4°C (Harlow *et al.*, 1988).

For live staining, the primary antibody was added directly to the parasite culture and incubated at 37°C for 1hr. Unbound antibody was removed by washing 3 times with TBS and the culture reconstituted with RPMI-A+. Secondary antibody was added, incubated, and rinsed in a manner similar to the primary antibody. The erythrocytes were then resuspended in TBS and visualized as for IFA assays.

TABLE 2 - ANTIBODY STAINING PROTOCOLS

PRIMARY ANTIBODY				SECONDARY ANTIBODY		
Antibody	Target	Dilution	Time	Antibody	Dilution	Time
1C4	Band 3	undiluted	60 min	1a. αmouse-biotin 1b. avidin-FITC 2. αmouse-TR	1/20 1/20 1/50	30 min 30 min 60 min
3H3	Band 3	undiluted	60 min	as above		
1F4	Band 3	undiluted	60 min	as above		
ATS	PfEMP1 Acid Tail	1/100	60 min	α rabbit-FITC	1/50	60 min
MBP-r179	PfEMP1 CIDR	1/100	60 min	1. αrat-FITC 2. αmouse-TR	1/50 1/50	60 min 60 min
Sin-1	oxidized human rbcs	1/1000	30min	αmouse-TR	1/50	30 min

COMPUTER ANALYSIS

The statistical analysis and plotting programs of Microsoft Excel version 7.0 were used for all binding assay and statistical analyses; NIH Image PC was used for PCR product quantification; Geneworks 2.0 for MacIntosh was used for sequence alignments.

RESULTS

A. *VAR* GENE EXPRESSION *IN VITRO* DURING CULTURE

It has been estimated that the surface expressed variant of PfEMP1 changes at a rate of 2.4% per generation during *in vitro* culture (Roberts *et al.*, 1992). It has also been estimated that *var* genes account for up to 6% of the parasite's genome. Given these observations, we hypothesized that the *var* genes expressed by a clone of *P. falciparum* would change in culture over time. To examine this, an RT-PCR strategy was developed. Generic primers UNIEBP5'/3' were used to amplify DBL domains from expressed *var* genes (Smith *et al.*, 1995). Since these primers also amplify the DBL domains of erythrocyte binding proteins *eba-175* and *ebl-1*, it was first necessary to determine whether these genes were expressed, and how to distinguish their amplicons from those of *var* genes.

Agarose gel electrophoresis revealed that RT-PCR of total RNA from an asynchronous ItG clone *P. falciparum* culture yielded two major amplicons, 300 bp and 450 bp (Figure 3A, lane 2). It has previously been reported that the smaller amplified product corresponds to both the *eba-175* and *ebl-1* amplicons (Peterson *et al.*, 1995). Restriction enzyme analysis of this product revealed that in the ItG clone used, *eba-175* is expressed since the amplicon was cleaved by Hinf I and Hind III as predicted by the DNA sequence. Dra I however, failed to cut the 300 bp band, as would be expected if *ebl-1* were present, indicating that this product is not expressed to appreciable levels in this ItG clone (Figure 3B). *ebl-1* was first described in 1995, and due to its identity to *eba-175*, is presumed to encode an erythrocyte binding protein. It has been shown to be a single copy gene, and actively transcribed in asynchronous cultures of the Dd2 and 3D7 isolates; transcription in the ItG clone has not previously been demonstrated.

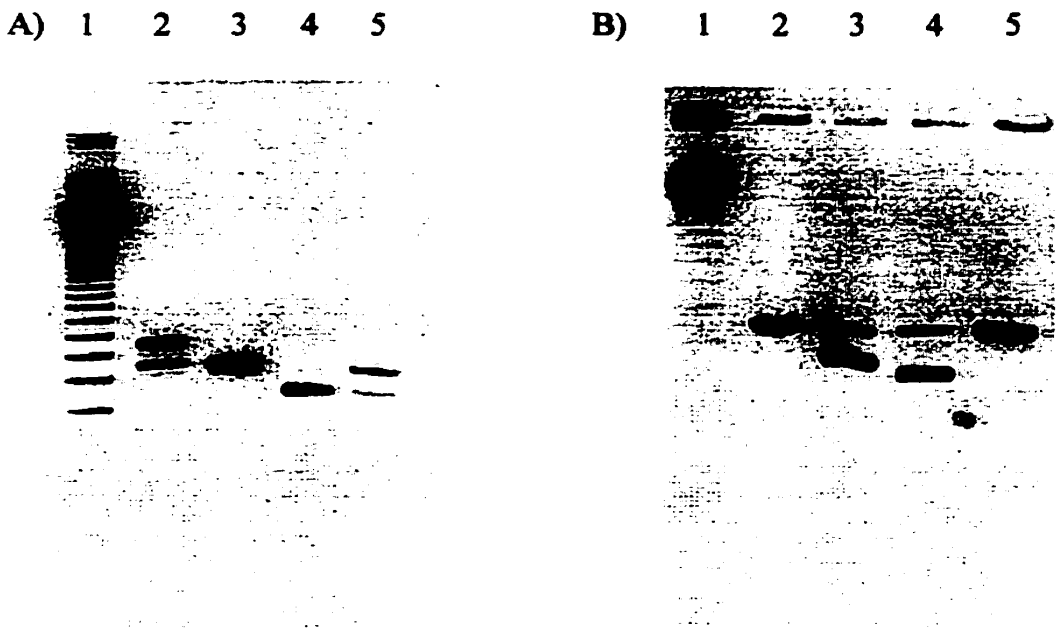


FIGURE 3 - *VAR* GENE AMPLIFICATION PRODUCTS
(A) RT-PCR Amplification products with primer pairs UNIEBP (lane 2), ACT (lane 3), ATS (lane 4), ACT/ATS (lane 5). 123 bp ladder (lane 1).
(B) Restriction Enzyme digestion of the 300 bp UNIEBP amplicon with no enzyme (lane 2), Hinf I (lane3), Hind III (lane 4) or Dra I (lane 5). 123 bp ladder (lane 1).
Figure shows the results of a representative experiment. Similar results were observed with 3 independent experiments.

To confirm that the 300 bp band does represent *eba-175*, it was excised, purified and subjected to SSCP. The UNIEBP5'/3' amplicon product of a cloned *eba-175* gene was also run concurrently for comparison purposes. The two amplicons gave similar SSCP patterns of a predominant DNA doublet (arrowhead), confirming that the 300bp amplicon was *eba-175* (Figure 4, lanes 2,3).

Culture samples collected weekly over a 2 month period were also subjected to RT-PCR and SSCP. The PCR product was used directly, or the larger band excised and gel purified before use. Figure 5 reveals the results of this SSCP. In relation to the *eba-175* doublet, both faster and slower moving variable bands were revealed.

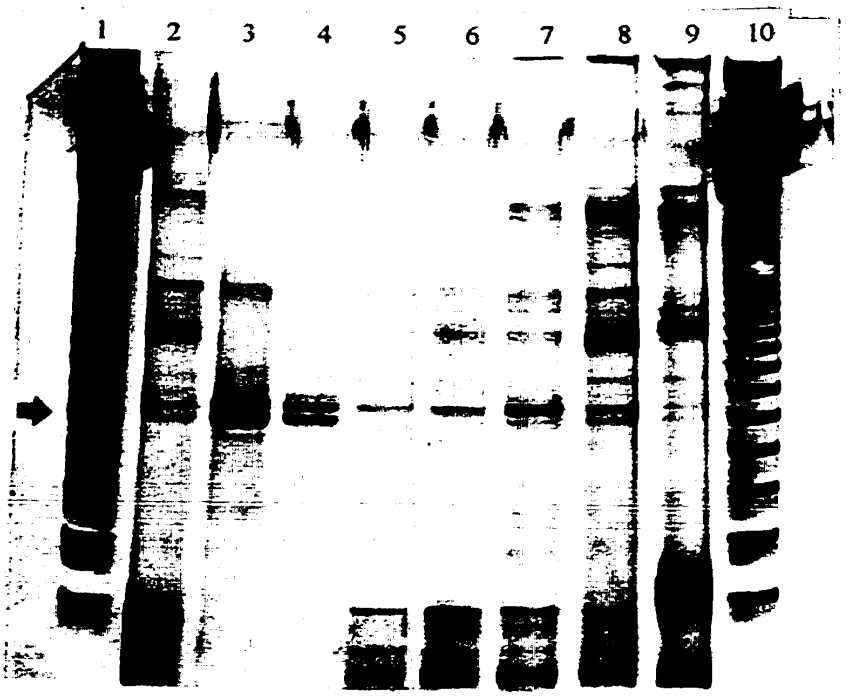


FIGURE 4 - SSCP OF DBL PCR AMPLICONS
UNIEBP amplicons of: ItG gDNA (lanes 2, 5-8), 300 bp band (lane 3), Wild isolate JP cDNA (lane 9). 123 bp ladder (lanes 1 & 10). 1200 Volt-Hours.

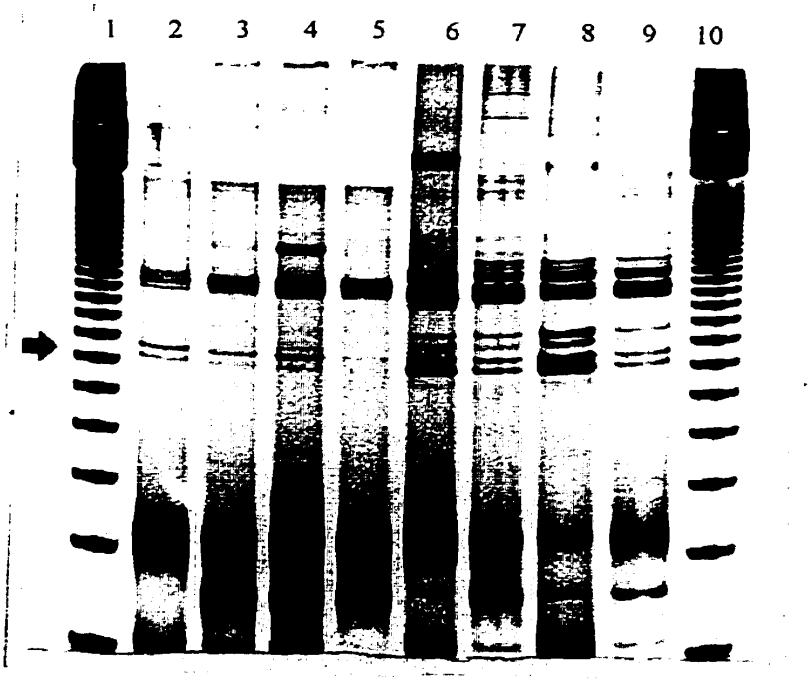


FIGURE 5 - SEQUENTIAL RT-PCR PRODUCTS
UNIEPB amplicons of RNA isolated on 30/4/97, 12/5/97, 15/5/97, 23/5/97, 27/5/97, 10/6/97, 23/6/97, 27/6/97 (lanes 2-9). 123 bp ladder (lanes 1 & 10). 700 Volt-Hours.
FIGURE 13 - DUAL LABELING OF ASYNCHRONOUS INFECTED ERYTHROCYTES

The presence of multiple bands suggests that several *var* genes are being expressed in a culture simultaneously. To confirm this, the RT-PCR DBL products from a synchronized culture were gel purified, TA cloned, and sequenced. Ten randomly chosen clones were examined resulting in 5 unique sequences. Extrapolating the results from this representative random population to the whole population of expressed DBL products the proportions (5:2:1:1:1) suggest that there were one or two predominantly expressed *var* genes in the culture at the point examined. The presence of multiple bands in excess of the *eba-175* doublet on SSCP analysis also suggests that there were several *var* genes expressed in culture at the time of analysis.

The pattern of these bands changed over time with a decrease in intensity of at least two bands in the faster moving group and an increase in the staining intensity and number of bands in the slower moving group. This suggests that there is a change in the expressed *var* genes with time.

When the SSCP pattern of the expressed DBL domains of a cultured wild isolate (Figure 4; lane 9) was examined, the two distinct groups of fast and slow moving bands were again seen. However, when compared to the ItG culture, the banding pattern is different, with slower moving products in both groups and a unique major product.

B. QUANTITATIVE RT-PCR

Although primers UNIEBP5'/3' were designed to amplify a wide variety of expressed DBL domains, they are degenerate primers and their corresponding target sequences contain considerable variability. Therefore it is possible that the amplicon pool they generate is biased. Thus, a primer pair was designed which would specifically amplify the products of

all expressed *var* genes. DNA sequences of 5 full-length *var* genes were aligned using Geneworks 2.0 for MacIntosh, and areas of identity were located. As previously reported, exon 2 sequences corresponding to the ATS are the most conserved, (Su *et al.*, 1995) and a primer pair to amplify a 150bp product from this region was designed (Figure 3A, lane 4). Once PCR conditions were optimized by adjusting primer pair concentration, and cycling parameters, products were PCR sequenced to verify that the primers were amplifying the correct target.

To evaluate the level of exon 2 expression, a quantitative RT-PCR strategy was developed. Since *Pf-actin1* has been shown to be expressed in all parasite stages, it was targeted as the internal reference standard (Wesseling *et al.*, 1989). Primers were designed to amplify a 300bp product, clearly distinguishable from the ATS product on agarose gel electrophoresis (Figure 3A, lane 4). These primers were designed to have a T_m identical to the ATS primer pair so that they were suitable for single tube amplification of both ATS and ACT products.

ATS and ACT primer pairs were then used in combination to optimize quantitative RT-PCR conditions (Figure 3A, lane 5). Testing was done by diluting 1μg of reverse transcribed total RNA from an asynchronous culture 1:2 in the working RT buffer. Cycling conditions and primer concentrations were then adjusted until the linear range of amplification was determined. To quantitate product amplification, constant volumes of PCR products were loaded onto agarose or polyacrylamide gels and stained with ethidium bromide or silver stain. Gel images were then digitized using a UVP Gel Documentation System GDS500 or a Hewlett Packard Scanjet 4c scanner and band densities determined using the PC version of NIH Image. It was found that 30 cycles of single tube amplification with

primers ATSF/R and ACTF/R at 0.5μM and 1.0μM respectively produced linear amplification with an input of 7.8 to 62.5ηg total RNA for the ATS product and 7.8 to 15.6ηg for the ACT product (Figure 6). This range of linear amplification was verified by independent RT-PCR of a new RNA sample.

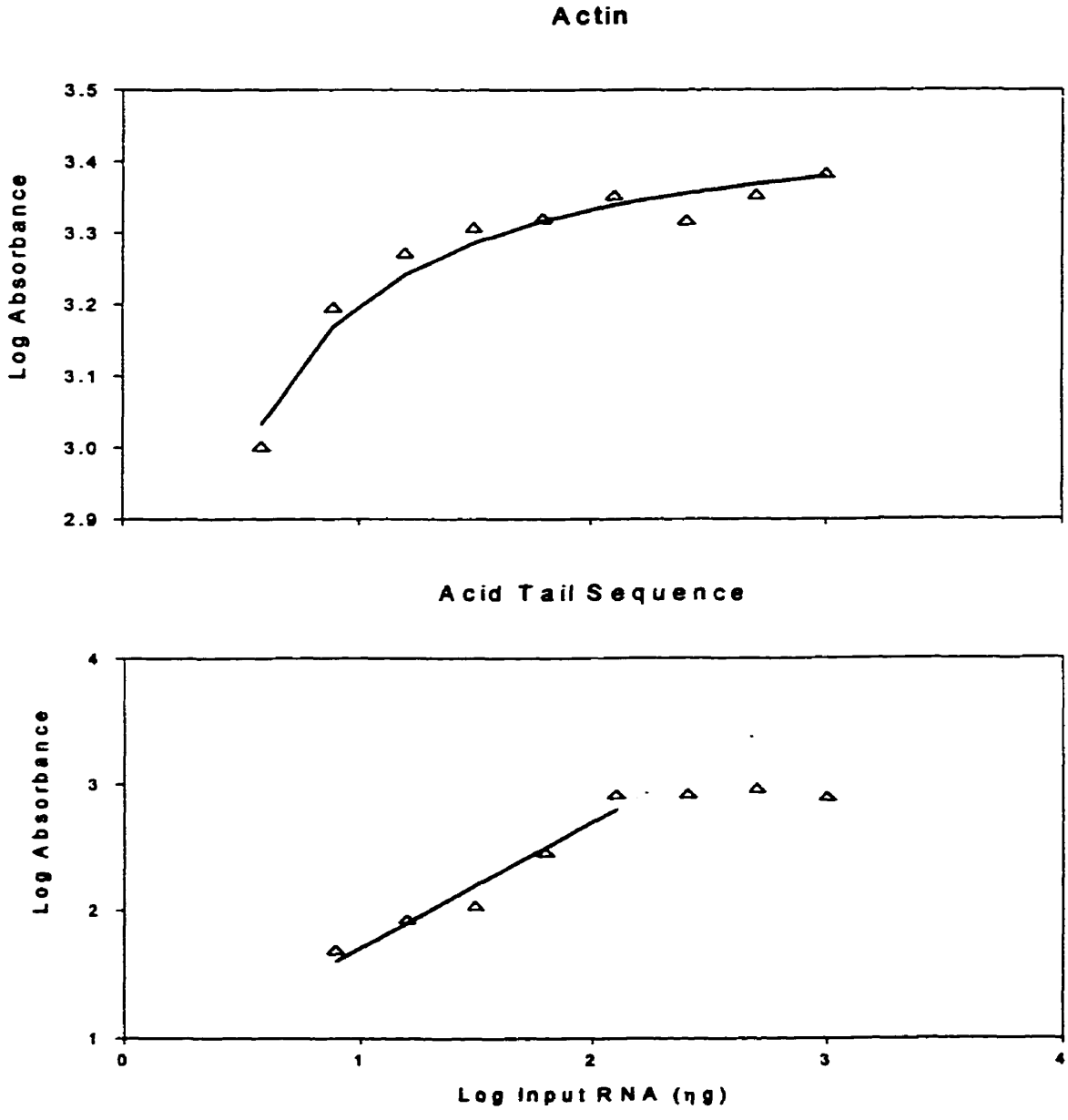


FIGURE 6 - VALIDATION OF LINEAR RANGE OF ATS AND ACT AMPLIFICATION
Exploration of linear range of amplification during single tube RT-PCR with primers ATSF/R and ACTF/R. Log input RNA (ηg) vs. Log absorbance

C. TIME COURSE ASSAYS

Each of the specific objectives examined in these studies attempts to relate a feature of cytoadherence to the developmental progression of the parasite. In an infected human, cyclic fevers help maintain IE synchrony (Gravenor and Kwiatkowski, 1998), but, in vitro, there are no such periodic fluctuations in temperature, and consequently, cultures are an asynchronous population of both ring and mature forms. Thus a prerequisite to temporal studies was the generation of a highly synchronous parasite population. If the sampling interval is longer than the difference in age between the youngest and oldest parasites in the culture, then the whole parasite population undergoes similar changes between intervals. Thus the behaviour of the culture approximates the behaviour of a single organism, allowing simple correlations between temporal events and developmental stage.

Stringent synchronization of parasite cultures was achieved as previously described using a combination of gelatin floatation and sorbitol lysis. This combined strategy yielded the most tightly synchronous cultures suited to rigorous temporal studies and allowed the most flexibility in the determination of the sampling interval. Although hourly sampling would have been ideal, limited erythrocyte and serum supply as well as the physical constraints on a single investigator made such frequent sampling impossible. Instead, the sampling interval was established at four hours, and the parasites were synchronized to be less than four hours divergent.

At each interval, cultures were sampled for parasite developmental stage, receptor binding, *var* gene expression and ligand surface expression. Six independent 48 hour time course studies and three 12 hour experiments, focusing on 12 to 24 hours post-invasion, were performed.

1. CULTURE SYNCHRONIZATION

Culture synchrony and parasite stage were assessed by microscopic examination of Giemsa stained thin smears. Although all *P. falciparum* parasites follow an identical asexual life cycle, which approximates 48 hours, the exact length of this cycle, and the proportion of it spent in the different developmental stages varies considerably from strain to strain (Lambros and Vanderberg, 1979). The ItG clone used in these experiments gave two patterns of stage distribution as shown in Figure 7. In both, the parasites exist as young rings with no thickening of the cytoplasm for the first 12 to 16 hours and as late rings, distinguished by a decrease in vacuole size and an increase in cytoplasm volume from 12 to 24 hours. The appearance in culture of the subsequent developmental stage, the trophozoite, in which the food vacuole is visibly replaced by cytoplasm, differs in the two patterns. In Pattern A, trophozoites are present in the culture beginning at 20 hours post invasion, and in Pattern B the emergence of this stage does not occur until 24 hours into the life cycle. Schizonts typically are present beginning 36 hours post invasion and remain in this form for 4 to 12 hours before rupturing. This translates into a life cycle of 40 to 48 hours.

Although there was some minor variation within these patterns, experiments 2, 5 and 6 exhibited Pattern A, and experiments 1,3 and 4 exhibited pattern B. The variation can be appreciated in Figures 8 and 9 which correspond to Pattern A, experiments 5 and 6. In experiment 5, 22% of the culture had entered the trophozoite stage by 20 hours and 18% of the culture persisted as late ring forms at 24 hours. In experiment 6, 28% of the culture had transformed to trophozoites at 20 hours and 10% of the culture remained as late rings at 24 hours. Similarly at 36 hours, 32% of the experiment 5 culture was in the schizont stage whereas only 10% of the culture in experiment 6 was recognized as schizonts at the same

relative time. Figure 10 represents Pattern B, experiment 3. In this experiment the parasites were in greater synchrony as witnessed by the low percentage of rings and trophozoites in the cultures at 24 and 40 hours respectively.

Figure 8 also demonstrates a divergence in culture synchrony with time. Although in all experiments parasites changed from early to late rings within 8 hours, (from 8 to 16 hours), it took 12 hours for the culture to complete the transition to trophozoites or schizonts, (from 16 to 28 hours, and 32 to 44 hours, respectively).

FIGURE 7 - PATTERNS OF DEVELOPMENTAL STAGE DISTRIBUTION IN THE ITG LIFE CYCLE
Patterns A and B represent the stage distribution of the ItG clone in culture as assessed by light microscopy. Experiments 2, 5 and 6 are represented by Pattern A and experiments 1, 3 and 4 are represented by Pattern B.

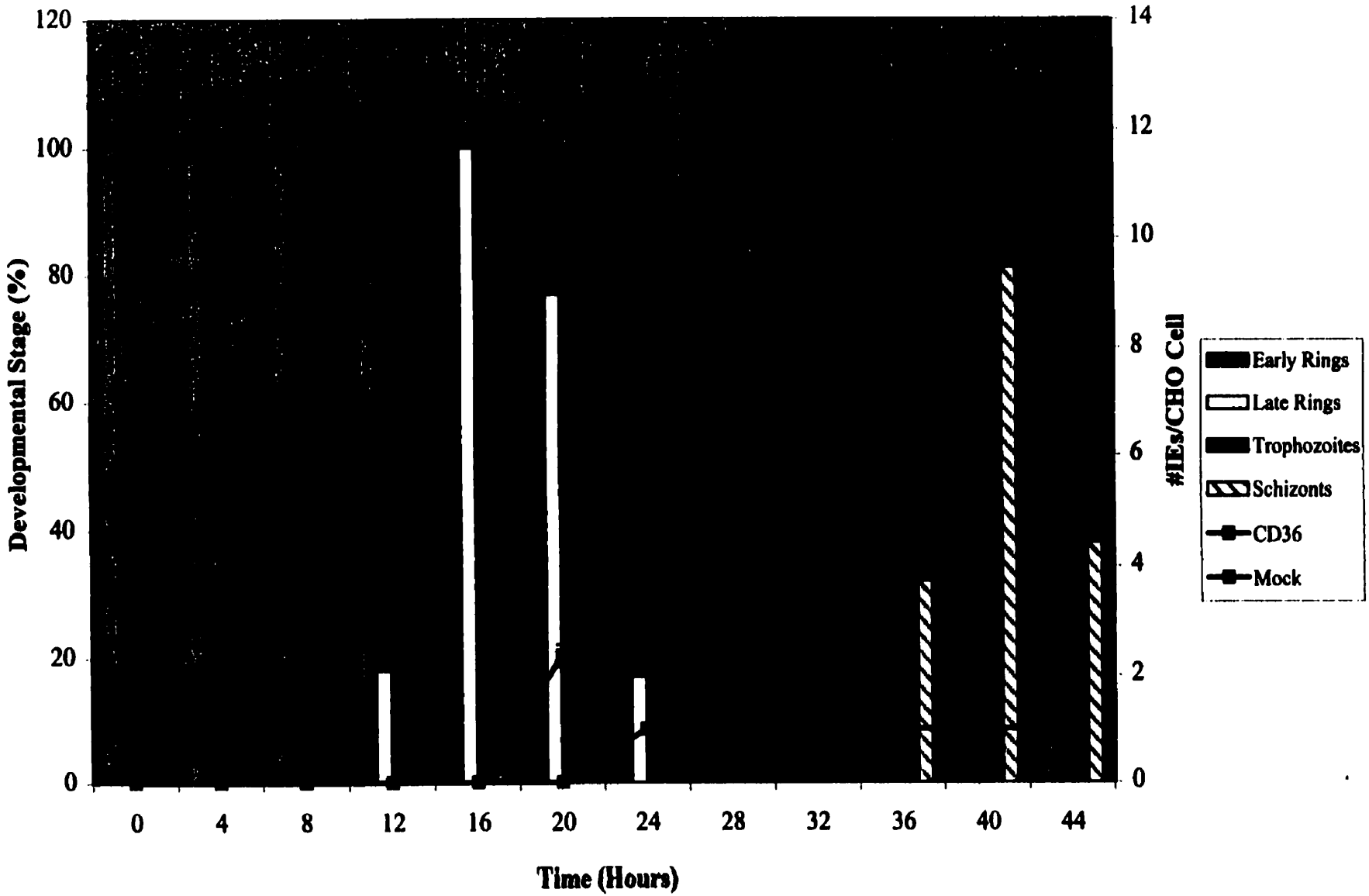


FIGURE 8 - TIME COURSE 5 - DEVELOPMENTAL PERCENTAGES AND CHO CELL BINDING

Bars - represents the percentage of the culture in each developmental stage. This was determined by counting 10 high power microscopy fields of Giemsa stained blood films taken every 4 hours through out the asexual lifecycle. **Lines** - represent IE binding to CHO-CD36 or CHOϕ (mock-transfected) cells. The mean number of adherent IEs was assessed by counting the number of IE associated with CHO target cells (a minimum of 50 target cells were counted).

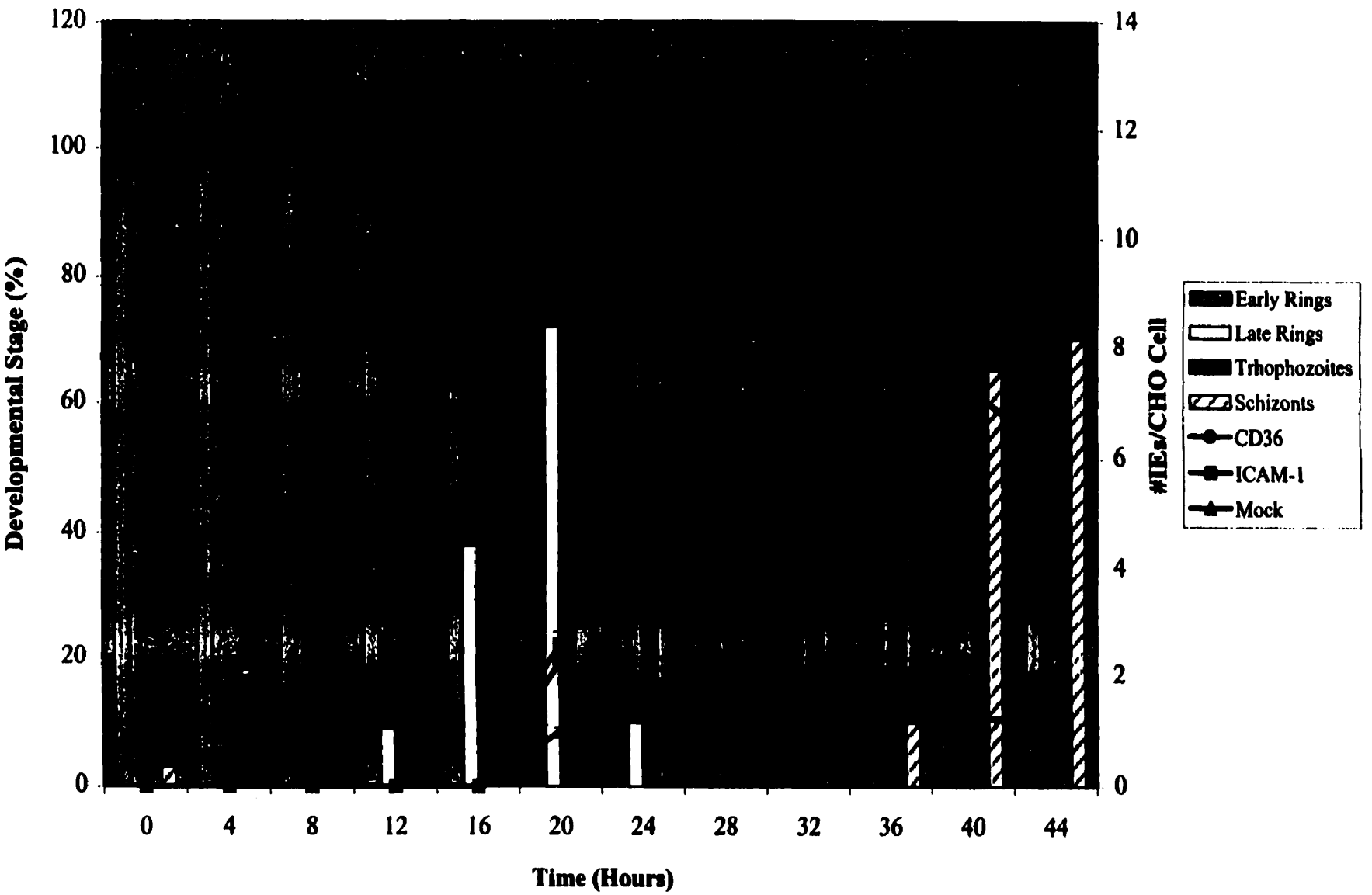


FIGURE 9 - TIME COURSE 6 - DEVELOPMENTAL PERCENTAGES AND CHO CELL BINDING

Bars - represents the percentage of the culture in each developmental stage. This was determined by counting 10 high power microscopy fields of Giemsa stained blood films taken every 4 hours through out the asexual lifecycle. **Lines** - represent IE binding to CHO-CD36, CHO-ICAM or CHOϕ (mock- transfected) cells. The mean number of adherent IEs was assessed by counting the number of fixed IE associated with CHO target cells (a minimum of 50 target cells were counted).

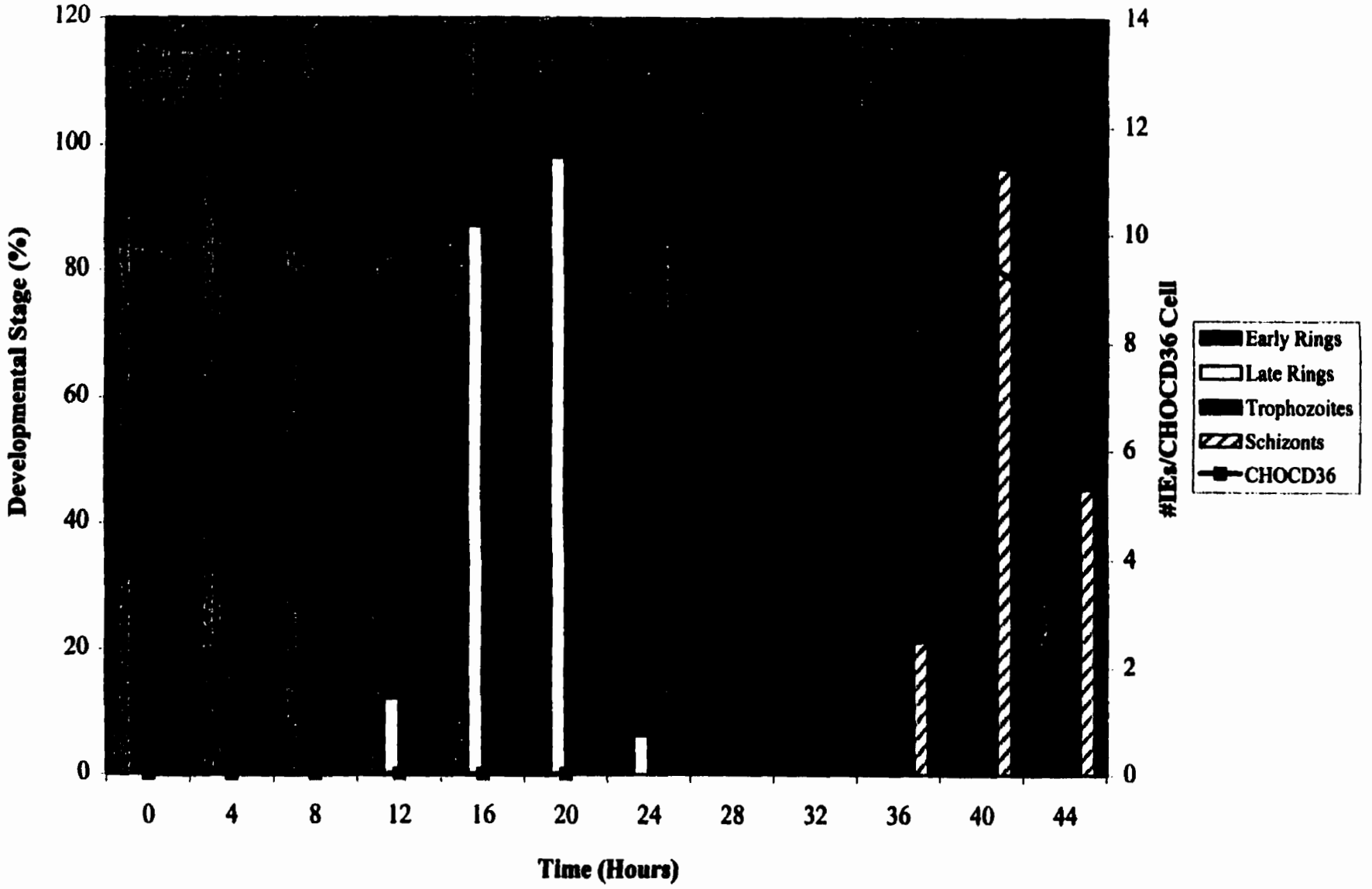


FIGURE 10 - TIME COURSE 3 - DEVELOPMENTAL PERCENTAGES AND CHO CELL BINDING
Bars - represents the percentage of the culture in each developmental stage. This was determined by counting 10 high power microscopy fields of Giemsa stained blood films taken every 4 hours through out the asexual lifecycle. **Lines** - represent IE binding to CHO-CD36 cells. The mean number of adherent IEs was assessed by counting the number of fixed IE associated with CHO target cells (a minimum of 50 target cells were counted).

2. BINDING ASSAYS

To examine the temporal onset of receptor binding, cytoadherence to CHO-CD36, CHO-ICAM or CHOϕ cells were performed. Since CD36 mediated cytoadherence is a consistent property of the ItG clone, the binding profile of this receptor was readily determined in all six time course experiments (Figure 11).

As expected, ring forms of the parasite were non-adherent; no binding was seen for the first 16 to 20 hours of the cycle. When the parasites passed into the trophozoite form, they became cytoadherent to CHO-CD36 and CHO-ICAM cells. Microscopically, this transformation is defined by the appearance of hemozoin pigment and the loss of resolution of the food vacuole in Giemsa-stained thin smears. Maximal levels of cytoadherence (mean = 9.11 ± 0.6, range 6.89 ± 0.39 to 10.56 ± 0.61 IEs/cell), were achieved coincident with the onset of binding, or at the succeeding time point in the assay. Thus IEs became cytoadherent to CHO-CD36 cells according to the stage distribution patterns seen in Figure 7, i.e. at 20 hours post-invasion in experiments 2, 5 and 6 and at 24 hours post-invasion in experiments 1, 3 and 4. The correlation between parasite stage and binding is most clearly shown in Figures 8 and 9 which represent experiments 5 and 6 respectively. Once maximal binding was reached, the level remained constant until schizont rupture.

Typically, ItG binding to the ICAM-1 is inconsistent and low (Biggs *et al.*, 1992). To increase ICAM-1 binding, parasites were panned on CHO-ICAM cells and the adherent population (ItG-ICAM) was expanded *in vitro*. This selection process was performed twice and CHO-ICAM binding was then assessed. Duplicate assays revealed that the ItG-ICAM parasites bound CHO-ICAM cells at significantly higher levels than the parental line ($p<0.0001$) (Table 3).

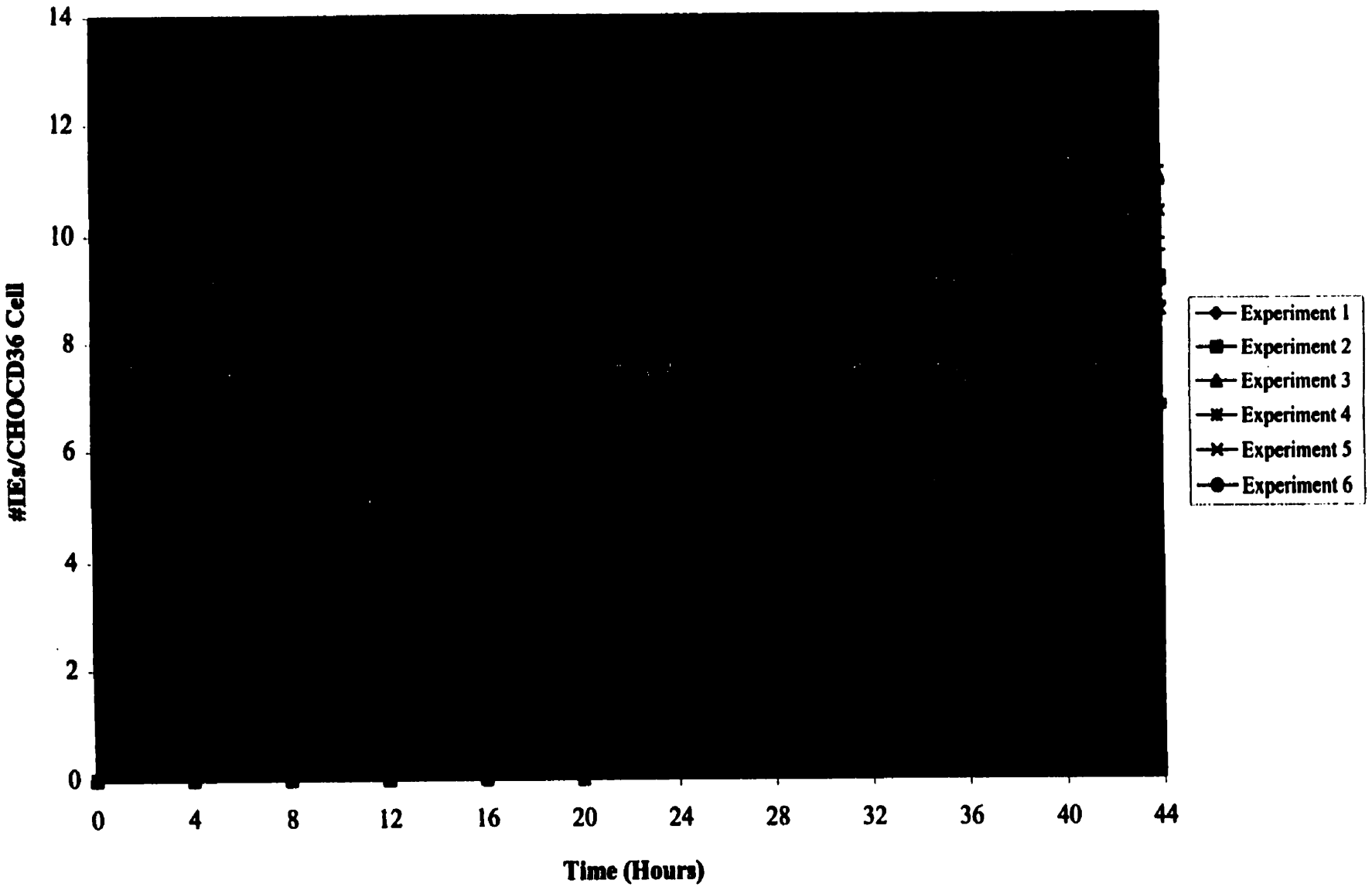


FIGURE 11 - CHOCD36 BINDING - EXPERIMENTS 1 TO 6
The number of adherent IE was assessed at 4 hour intervals. The mean number of adherent IEs per CHO-CD36 cell was assessed by counting the number of fixed IE associated with a minimum of 50 target cells.

PARASITE	#IE/CHO-CD36 CELL	#IE/CHO-ICAM CELL	#IE/CHOϕ CELL
ItG (parent)	11.36 ± 4.97	3.10 ± 1.45	1.1 ± 0.36
	10.94 ± 3.31	2.26 ± 0.66	1.1 ± 0.30
ItG-ICAM	8.62 ± 3.29	8.56 ± 3.77	2.20 ± 0.73
	11.76 ± 3.67	9.42 ± 3.67	2.96 ± 1.04

TABLE 3 - CYTOADHERENCE OF PARENT ITG AND ITG-ICAM LINES
Following two rounds of panning on CHO-ICAM cells, cytoadherence to CHO-CD36, CHO-ICAM and CHOϕ was assessed. Asynchronous cultures containing ~5% mature form parasites i.e. trophozoites or schizonts were used for both experiments.

These parasites were used in time course studies 5 and 6, and produced temporal ICAM binding profiles similar to that of CD36 (Figure 9). Parasites became cytoadherent as trophozoites appeared in culture 20 hours post-invasion, and once maximal binding was reached, (3.77 ± 0.36, 6.82 ± 0.52), the level remained constant until schizont rupture began.

To ensure that binding to CHO-CD36 and CHO-ICAM cells was attributable to the respective receptors and not to another cell surface molecule expressed on the transfected CHO cell line, binding to CHOϕ cells was assessed in experiments 5 and 6. In both cases a small number of IEs bound to CHOϕ cells. This binding showed a significant increase in the selected ItG-ICAM line ($p<0.0001$). Since this binding was not observed with immature stages, it was presumed to be specific IE cytoadherence.

3. *VAR* GENE EXPRESSION

To examine PfEMP1 production, *var* gene expression was analyzed over time. Using the quantitative RT-PCR strategy, it was evident that *var* genes are maximally expressed 4 to 8 hours before the onset of binding, during the late ring stages (Figure 12). At all other times in the life cycle, low levels of *var* gene transcription occur. Since actin expression appears to vary with time it is not an ideal internal control. This result, coupled with the small amounts

of total RNA yielded in time course experiments made it difficult to obtain truly quantitative data, and limited analysis to experiments 1 and 2.

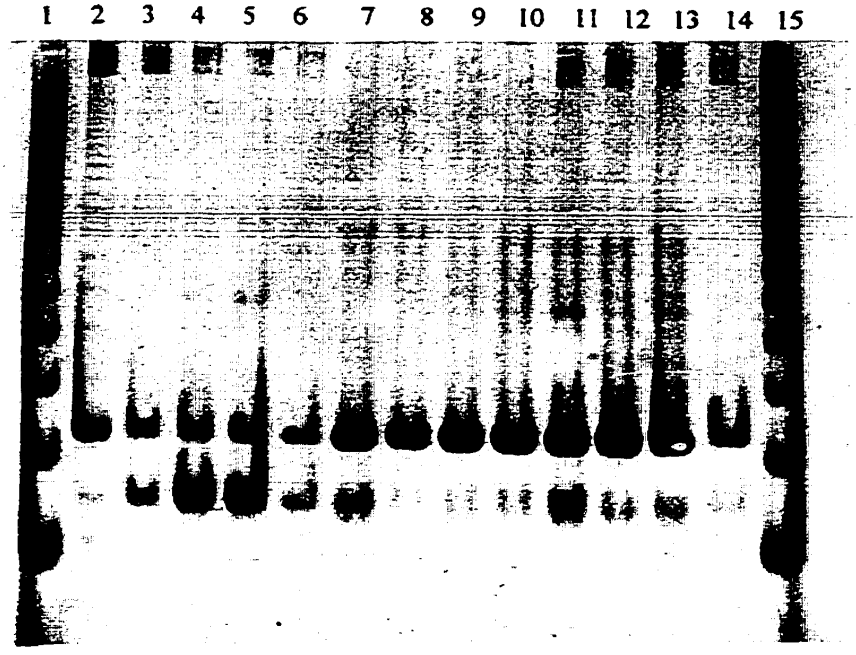


FIGURE 12 - TEMPORAL PATTERN OF *Pfactin I* AND *VAR* GENE EXPRESSION
RNA samples from experiment 2 were reverse transcribed and amplified using the ATS and ACT primer pairs. Lanes 1 & 15; 123 bp marker. Lanes 2-14: 0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48 hours respectively. The slower band corresponds to ACT product and the faster band corresponds to ATS.

4. IMMUNOFLUORESENCE

Since PfEMP1 and Band 3 neoantigens represent the two most studied ligands for CD36, their temporal synthesis and erythrocyte surface distribution were examined by immunofluoresence and confocal microscopy.

a. Dual Labeling

To examine whether several putative ligands are present simultaneously on a single infected cell, dual labeling of mature IEs was carried out. In control studies to validate the sequential indirect IFA staining it was found that a) rat polyclonal serum prepared against PfEMP1 (MBP-r179) is recognized by the goat anti-mouse IgG normally used to detect band 3 neoantigen and b) epitope saturation of the primary antibody with the fluorescent tagged secondary antibody is incomplete. For these reasons, it was necessary to directly label mAb 1C4 with FITC. Dual labeling was then carried out using a layered protocol; the primary antibody of interest was detected with goat anti-mouse IgG-Texas Red, and 1C4-FITC staining was carried out last. This ensured a unique epitope specificity for each fluorescent tag used.

Mature stage parasites from asynchronous cultures were stained as described above and examined in three independent experiments. It was found that most IEs display each of the following epitope pairs: band 3 neoantigens 3H3 and 1C4 (Figure 13A), band 3 neoantigens 1F4 and 1C4 (Figure 13B) as well as PfEMP1 and band 3 neoantigen 1C4. (Figure 13C) However, the patterns of red and green florescence overlapped minimally suggesting that these antigens have distinct surface distributions.

b. Sin-1 Polyclonal Serum Validation

In order to confirm the surface localization of the various band 3 neoantigens and PfEMP1 epitopes, a reference surface marker was required. Human red cells were subjected to peroxynitrite oxidation with SiN-1 and were used to immunize mice (Dr. I. Crandall). The resultant polyclonal serum, referred to as Sin-1 was used at high dilutions (1/1000), to

avidly agglutinate live human A+ cells (Figure 14A). Live or fixed individual RBCs show a pattern of surface florescence when stained with Sin-1 sera indicating that this antisera recognizes the erythrocyte surface (Figure 14B). Furthermore, confocal cross section data using sin-1 serum, clearly depict the concave shape of the RBC membrane (Figure 14C).

c. Altered Band 3 Neoantigens

Multiple band 3 neoantigens have been detected in IEs, a subset of which have been implicated in cytoadherence (Crandall and Sherman, 1994a; Guthrie *et al.*, 1995). In these studies, the epitopes detected by mAb 1F4, 3H3 and 1C4 were examined for each 48 hour time course series and the more focused 12 hour time course experiments.

The band 3 neoantigen recognized by monoclonal antibody 1F4 was detected on IEs as early as 12 hours post-invasion i.e. early rings (Figure 15A). The persistence of this epitope for the remainder of the life-cycle was inconsistent. Using the z-scan function of the Carl Zeiss confocal microscope, vertical cross sections were generated through the IE. These images revealed that this epitope is present on the surface of the IE (Figure 15B). The band 3 neoantigen recognized by monoclonal antibody 3H3 was also expressed on the IE surface in an uneven pattern beginning at 12 hours post-invasion (Figure 15C, 15D). The uneven staining pattern observed persisted for the remainder of the life cycle.

The remaining band 3 epitope examined was recognized by the mAb 1C4. IEs were first recognized by the primary antibody at 20 or 24 hours, coincident with the onset of binding and the pattern of developmental stage outlined in Figure 7. i.e. fluorescence was observed in thin smears made at 20 hours for experiments 2, 5 and 6 and in thin smears made at 24 hours in experiments 1, 3 and 4 (Figure 15E). The pattern of fluorescence was

punctate and persisted for remainder of the life cycle. Cross sectional images reveal that this epitope is also surface expressed (Figure 15F).

d. PfEMP1

PfEMP1 synthesis and surface distribution was examined in the abbreviated 12 hour time course assays which focused on the period from 12 to 24 hours post-invasion. Two polyclonal sera were used to examine this protein. The ATS rabbit serum recognizes the conserved cytoplasmic tail of the protein, and the MBP-r179 rat sera, an extracellular domain believed to be involved in CD36 binding (Reeder *et al.*, 1997; Baruch *et al.*, 1997).

Staining with MBP-r179 revealed that PfEMP1 begins to accumulate in IE during the late ring stage, 8 hours prior to binding. Since binding began at 20 hours post-invasion in each of these experiments, this means that fluorescence was detected in fixed thin smears at 12 hours post-invasion. At this point, an intense ring of immunostaining is present, likely corresponding to the parasite cytoplasm (Figure 16A, 16B). Four hours prior to binding, the pattern of immunostaining is very different. Foci of fluorescence are scattered in a single area and are confined inside the IE membrane (Figure 16C, 16D). Coincident with the onset of binding, MBP-r179 stains the IE diffusely over its surface, although some areas of localization are apparent (Figure 16E). Again, z-scan confocal microscopy confirmed the presence of this protein on the IE surface (Figure 16F). This temporal appearance and of PfEMP1 and its cellular distribution was confirmed with the ATS sera.

e. Live Staining

To independently confirm the temporal surface localization of the band 3 neoantigen recognized by the monoclonal antibody 1C4 and PfEMP1, live synchronous cultures from the focused 12 hour experiments were stained with mAb 1C4 or MBP-r179. The staining protocol used was similar to that for fixed thin smear IFA (detailed in Table 2), except that a higher concentration of the primary antibodies was used since larger volumes of erythrocytes were being stained. Additionally, TBS washes were carried out in microfuge tubes, and incubations were performed at 37°C to maintain culture viability.

In agreement with confocal cross section data, both the band 3 neoantigen and PfEMP1 were surface expressed at 20 hours, coincident with the onset of binding (Figure 17A, 17B).

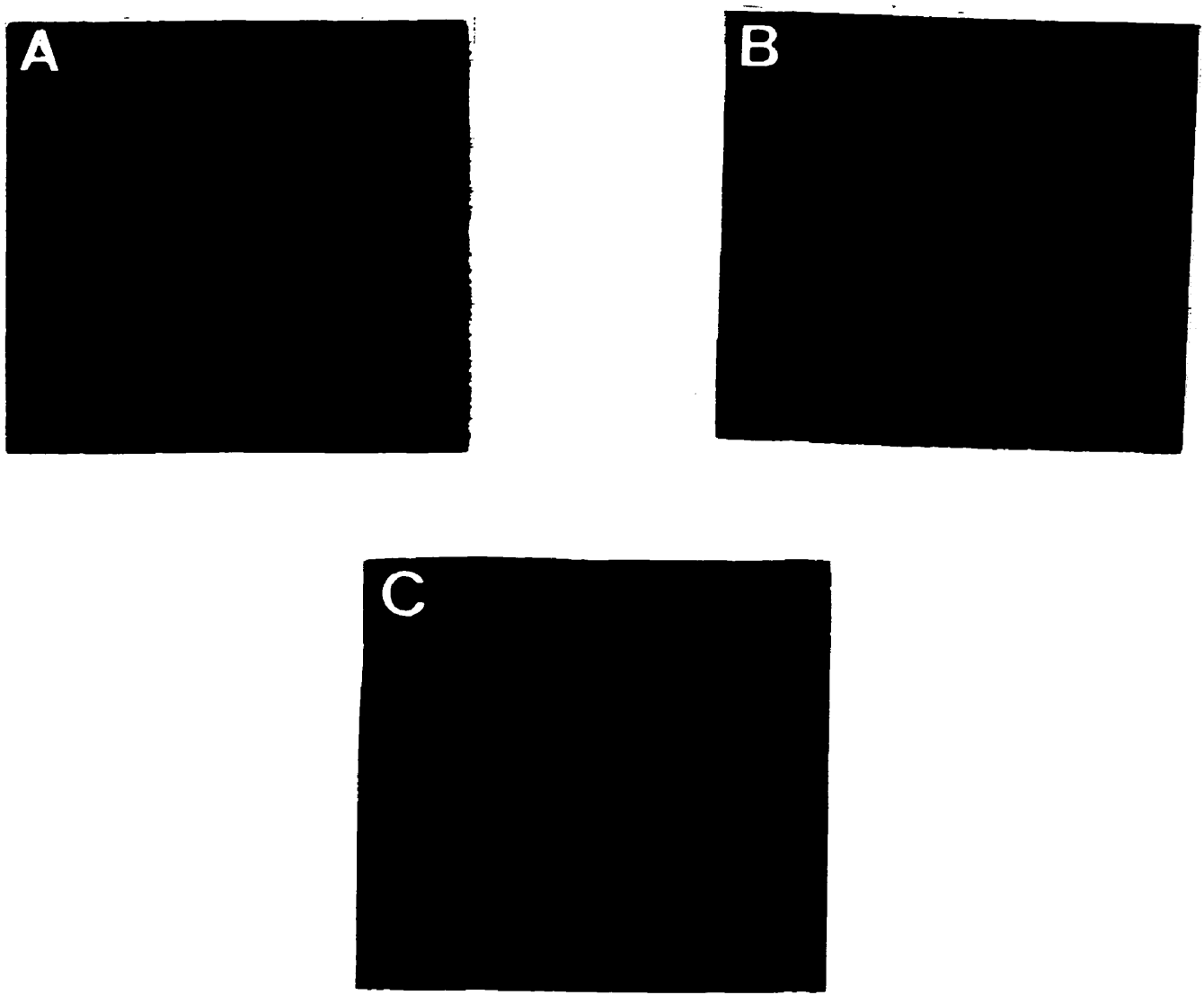


FIGURE 13 – DUAL LABELING OF ASYNCHRONOUS INFECTED ERYTHROCYTES
Fixed thin smears of asynchronous parasite cultures were stained with:
Band 3 neoantigen 3H3 (red) and band 3 neoantigen 1C4 (green). (A)
Band 3 neoantigen 1F4 (red) and band 3 neoantigen 1C4 (green). (B)
PfEMP1 (red) and band 3 neoantigen 1C4 (green). (C)

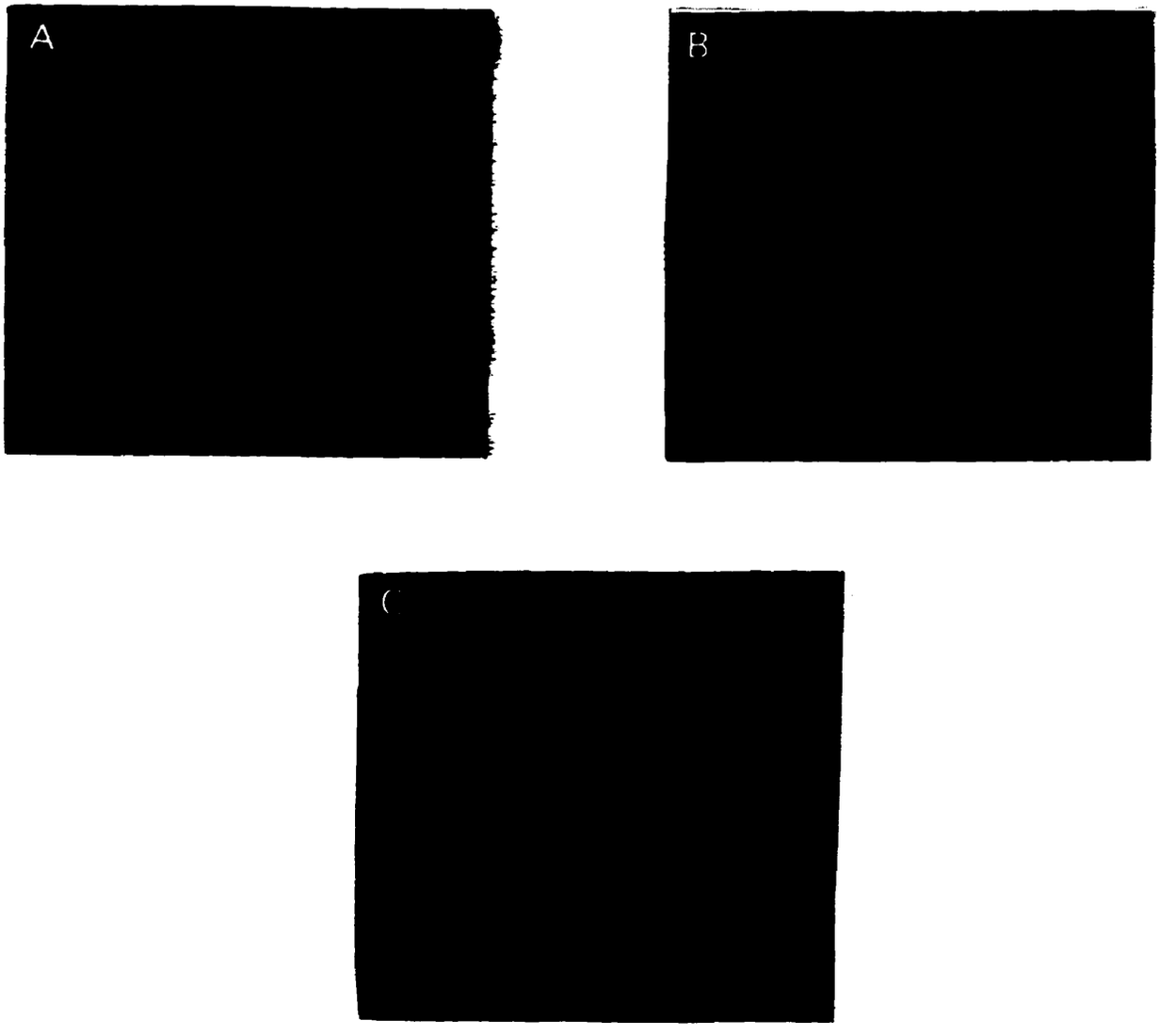


FIGURE 14 - POLYCLONAL SIN-1 STAINING
Live erythrocytes were stained with Sin-1polyclonal serum and anti-mouse TR:
Agglutination was observed both macroscopically and microscopically. (A)
Single cells demonstrated peripheral fluorescence. (B)
Fixed thin smears were then stained as above, and single, fluorescent cells analyzed by z-scan to give cross sectional views. Fluorescence is clearly surface associated in accessible areas. (C)

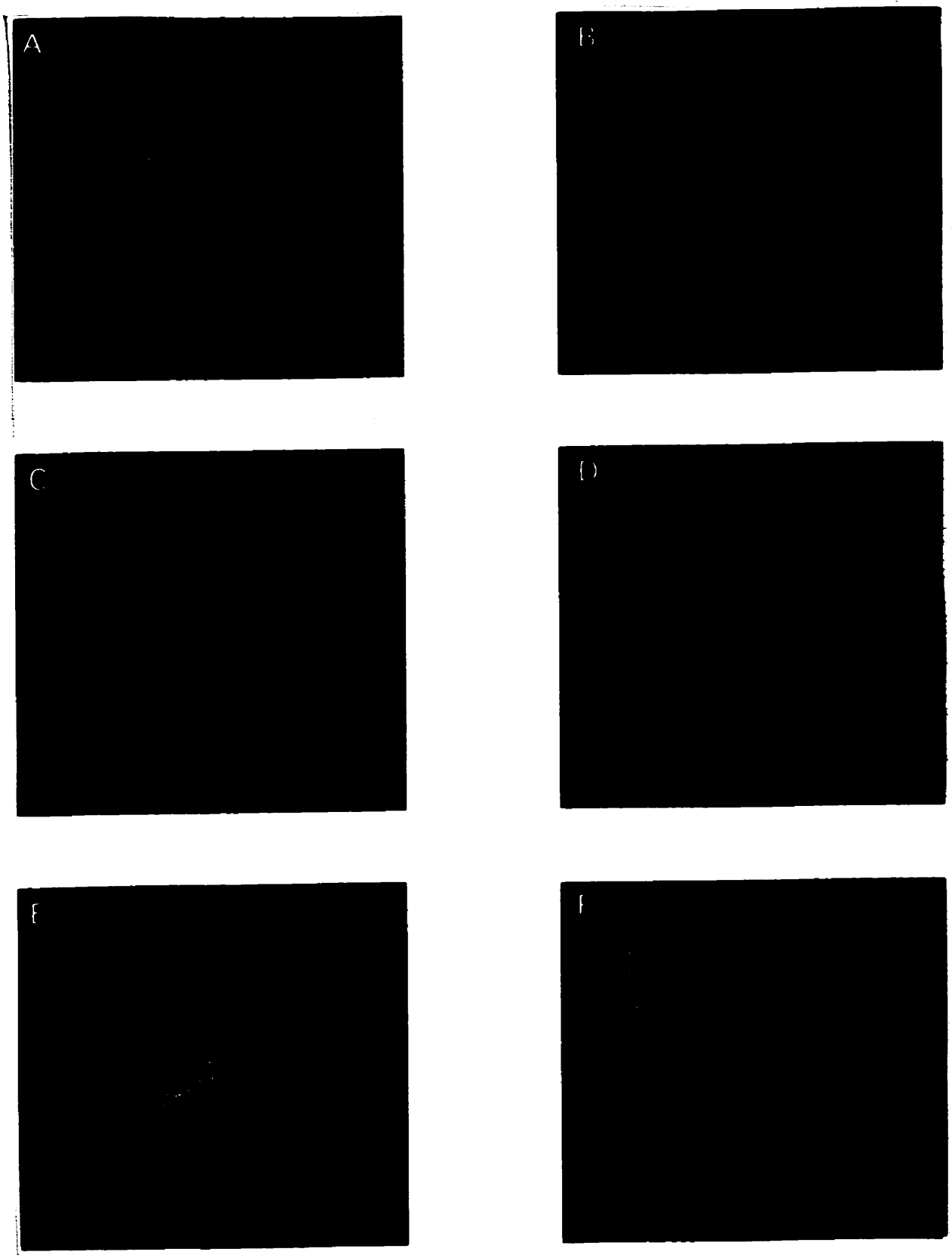


FIGURE 15 - BAND 3 NEOANTIGEN SURFACE EXPRESSION
Fixed thin smears were stained with an anti-band 3 mAb and anti-mouse-fluorescein conjugate (green), and counterstained with Sin-1 polyclonal serum and anti-mouse TR to define the cell surface. (red)
Panels A, C and E are transverse views. Panels B, D and F represent cross-sectional views generated by z-scan. Band 3 neoantigen 1F4 (Panels A, B), Band 3 neoantigen 3H3 (Panels C, D), Band 3 neoantigen 1C4 (Panels E, F).

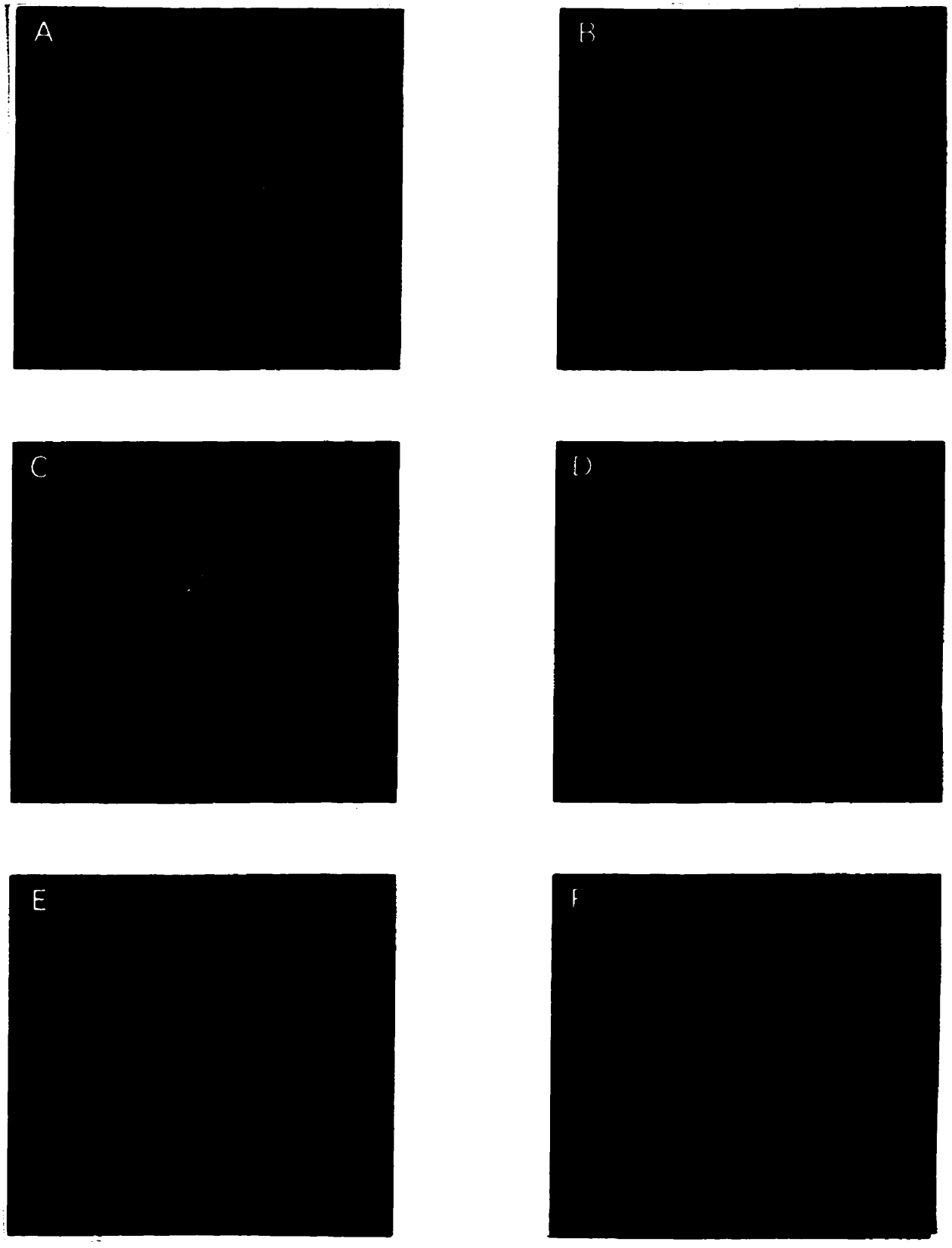


FIGURE 16 - PfEMP1 SURFACE EXPRESSION
Fixed thin smears were stained with polyclonal serum MBP-r179 which recognizes PfEMP1 and anti-mouse-fluorescein conjugate (green), and counterstained with Sin-1 polyclonal serum and anti-mouse TR to define the cell surface. (red) Panels A, C and E are transverse views. Panels B, D and F represent cross-sectional views generated by z-scan. 8 hours prior to the onset of binding (Panels A, B). 4 hours prior to the onset of binding (Panels C, D). Coincident with the onset of binding (Panels E, F).

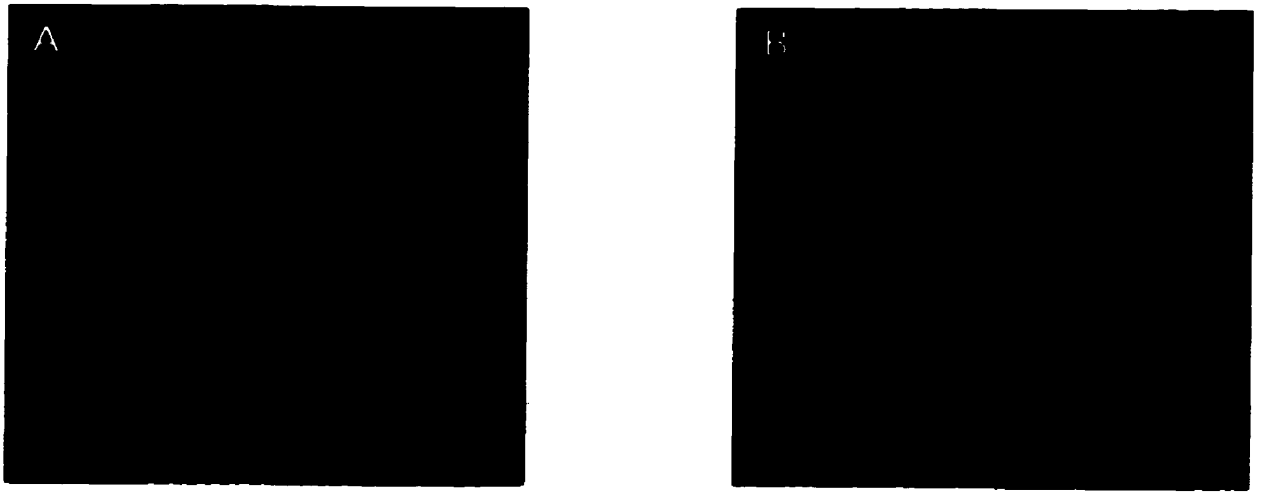


FIGURE 17 - LIVE INFECTED ERYTHROCYTE STAINING
Live cultures were stained with mouse monoclonal antibody to detect band 3 neoantigen 1C4 (A) or rat polyclonal serum MBP-r179 to detect PfEMP1 (B). Surface fluorescence was detected coincident with the onset of binding.

DISCUSSION

Each of the specific objectives examined in these studies related some feature of cytoadherence to the developmental progression of the parasite. Although the experimental design was to generate parasites that were tightly synchronized, this synchrony was not maintained through out the life cycle. Asynchrony could have been due to differential growth rates of individual parasites caused by differences in nutrient availability in multiply infected erythrocytes, older erythrocytes and other unknown influences. As the parasites become less synchronous, the assumption that the parasites are acting as a single organism is negated, and the correlation between observed changes and parasite developmental stage is confounded. Fortunately, the important temporal events related to cytoadherence observed in these studies occurred in the first half of the life cycle, so this differential growth effect had only a minor confounding influence on the results.

An additional complicating factor was the lack of consistent correlation of developmental stage with time. This limitation is related to the synchronization protocol and convention of assigning time zero as the moment of invasion. During synchronization, mature schizonts ruptured during a four hour incubation. The time at which the majority of schizonts ruptured during this incubation varied with each experiment. Since time zero was taken to be the termination of this phase, parasites in some experiments may actually have been up to 4 hours post-invasion at the beginning of the experiment. This inconsistency coupled with differential growth rates might account for the two patterns of developmental stage distribution outlined in Figure 7.

In a similar time course study conducted by Marsh and colleagues, it was suggested that the parasite developmental stage rather than time post-invasion is a more reliable

reference in defining the temporal onset of binding (Marsh *et al.*, 1988). The lack of a consistent correlation of developmental stage with time in these experiments supports this contention. Thus to avoid these confounding factors, parasite developmental stage, as assessed by light microscopy, rather than parasite age post-invasion, will be used as the reference in defining the various features of cytoadherence studied.

The first specific objective of these studies was to determine the temporal onset of IE binding to CD36 and ICAM-1. It was found that binding to both CD36 and ICAM-1 was closely linked to parasite phenotype. As the parasites passed from the late ring to trophozoite form, they became adherent to both receptors. Thus, within the limits of resolution of this experimental design, the ability of IEs to adhere to CD36 and ICAM-1 appears to be an off/on event. Since these experiments were performed under conditions of minimal shear stress, the pattern of adherence observed also appeared to be off/on. However, under flow conditions or *in vivo*, this off/on binding ability might not translate into a simple off/on pattern of adherence due to increased levels of shear stress, and the presence of multiple IE receptors.

Since the onset of binding closely correlated with parasite developmental stage, this time point also serves as a reliable reference in these studies. Although the onset of binding to the two receptors appeared to be coincident, it must be remembered that the sampling interval and the progressive desynchrony of the culture limit the resolution of the experiment such that adherence to the two receptors may only be closely approximately rather than exactly coincident.

Since the cultures used in these experiments represent a population of parasites, this off/on binding ability translates into an S-shaped binding profile. The parasites were

originally synchronized within four hours, and therefore the vertical grade of the curve should ideally be contained between two consecutive time points, as it is in Figure 10. Practically though, it often takes longer for maximal binding to be achieved as seen in Figures 8 and 9. This delay is attributable to a slight divergence in parasite growth rates, and the relative insensitivity of the four hour sampling interval for determining the exact degree of this divergence i.e. 5 hours vs. 8 hours. Once maximum binding levels were reached, 9.11 ± 0.6 IEs/cell per CHO-CD36 cell and 6.82 ± 0.52 IE per CHO-ICAM cell, they were maintained for the remainder of the life cycle until schizont rupture.

These results are, for the most part, consistent with other temporal binding studies (Marsh *et al.*, 1988; Treutiger *et al.*, 1998; Gardner *et al.*, 1996). In the Marsh study, the binding of a Gambian wild isolate to C32 melanoma cells was examined. Peripheral blood was drawn, placed *in vitro* culture and the onset of cytoadherence was at 20 to 24 hours post-culture initiation, coincident with the appearance of trophozoites. Once maximal binding was achieved at 28 hours post culture, levels remained constant until schizont rupture (Marsh *et al.*, 1988).

In another study, binding of the $A4^{tr}$ clone to purified CD36 and ICAM-1 was assessed (Gardner *et al.*, 1996). A culture, originally synchronized to be <1hr divergent, became cytoadherent to both receptors coincidentally at 16 hours post-invasion, and reached a stable maximum binding levels by 18 hours post-invasion. Microscopic data relating to parasite stage were not provided, so it is unclear if the discrepancy in the onset of binding relative to our results was solely due to differences in synchronization protocols and time point references or to clone differences.

A more recent study explored the binding of the FCR3 clone to C32 melanoma cells. Similar to the 1996 Gardner *et al.* study, the onset of binding was early; 12 hours post-invasion. However, microscopic data indicate that this is when the ring to trophozoite transition occurs in this clone. Moreover, culture synchronization in this experiment was not very stringent so time post-invasion is an unreliable reference. Additionally this group reported that it took 16 hours to reach maximal binding, which was not maintained for the remainder of the life cycle. This discrepancy may be due to the divergence in parasite age. However, C32 melanoma cells express many receptors including CD36, ICAM-1, TSP and CSA. The crescendo-decrescendo binding seen in this experiment may have been due to multiple IE ligands binding to TSP and CSA receptors absent in our experiments.

Our original hypothesis was that if either receptor plays a pivotal role in sequestration, it must support binding *in vitro* of mature IE at high levels for the duration of their presence in culture. Since both receptors satisfy these requirements, neither can be deemed from these studies to contribute more significantly to IE binding, or to play a more important role in sequestration *in vivo.*

However, consistent with the current hypothesis that IE binding to ICAM-1 has a fast off rate, (Craig *et al.*, 1997), is the observation that a special binding assay with lower shear stress had to be developed to assess ICAM-1 binding. *In vivo*, this fast off rate would likely lead to IE rolling rather than static adherence, as has been observed in *ex vivo* flow systems (Cooke *et al.*, 1994; Udomsangpetch *et al.*, 1997). Thus, our studies support the leukocyte recruitment based model of cerebral sequestration in which parasites roll on certain receptors including ICAM-1 facilitating capture by and static adherence to other receptors (Cooke *et al.*, 1994; Udomsangpetch *et al.*, 1997).

Another interesting observation revealed by these temporal binding studies is the presence of another IE specific receptor on both CHOϕ and CHO-ICAM cells. Panning of the parental ItG clone on CHO-ICAM cells significantly increased IE binding ($p<0.001$). This increase was originally attributed solely to ICAM-1, however, when binding to CHOϕ cells was assessed it was also elevated. ($p<0.001$). This elevation indicates that CHO-ICAM and CHOϕ both possess a common receptor on which IE can be panned to increase their binding phenotype. The ability of IE to bind to this unknown receptor shows the same off/on temporal profile as CD36 and ICAM-1, supporting the specificity of this receptor in IE binding. Chondroitin sulfate A, another proposed IE receptor, has been demonstrated to be present on CHO cells, and although it does not contribute significantly to cytoadherence in unselected parasite lines, it will bind panned IE, and may represent the unknown receptor in these studies (Rogerson *et al.*, 1995). Alternatively, an unknown IE receptor on CHO cells has recently been reported and may account for this increase in binding (Ho *et al.*, 1998).

Thus the ability of IE to adhere to the endothelial cell surface receptors examined appears to be an off/on event closely linked to the ring to trophozoite transition. Although this is consistent with clinical findings where mature IE are mostly lacking from the peripheral circulation, it is harder to reconcile with the current hypotheses that there are multiple IE ligands. How could the surface expression of multiple IE proteins be coordinated to result in off/on ability to bind to two distinct receptors? It is also difficult to reconcile off/on binding ability with the existence of receptor specific ligands since the parasites appeared to become cytoadherent to CD36 and ICAM-1 and the unknown receptor simultaneously.

The second specific objective of this study was to examine the temporal relationship between the surface expression of PfEMP1 and band 3 neoantigens and cytoadherence. Since binding to CD36 and ICAM-1 is an off/on event associated with parasite stage, those proteins intimately involved in adherence to these receptors should be surface expressed coincidentally with the onset of binding. Proposed ligands which appear on the surface prior to the onset of binding are not involved in adherence to these receptors, or require further modification or the presence of a cooperative molecule before participating in adhesion. Additionally, since maximal IE binding was rapidly achieved and maintained for the remainder of the life cycle, any proposed IE ligand surface expressed after the onset of binding is likely not involved in adherence to CD36 or ICAM-1.

Two of the band 3 neoantigens, those recognized by monoclonal antibodies 3H3 and 1F4, were consistently surface expressed on the ring stage IE, up to 12 hours before the onset of binding. In contrast, the band 3 epitope recognized by the monoclonal antibody 1C4 was consistently surface expressed at the ring to trophozoite transition.

Although the early appearance of band 3 neoantigens recognized by monoclonal Abs 3H3 and 1F4 indicate that their early conformations are incompatible with binding, it is possible that further degradation of the band 3 molecule might result in additional conformational changes, allowing these epitopes to form the more temporally relevant 1C4 neoantigen.

To examine this, mature IE were dually stained with mAb pairs recognizing 1C4 and one of 3H3 or 1F4. It was found that both the 3H3 and 1F4 epitopes persisted after the appearance of the 1C4 neoantigen, and that their distribution on the cell did not colocalize

with 1C4. Thus, 3H3 and 1F4 epitopes are distinct from the 1C4 neoantigen, and alone would not appear to contribute to cytoadherence.

How then do mAb that recognize these epitopes block cytoadherence? The most reasonable explanation would be that they sterically hinder a true ligand in close proximity on the IE membrane. This is hard to reconcile with the apparent distinct distribution of the epitope recognized by mAb 1C4, and suggests that some other mechanism, such as cross-reaction of the mAbs with an active ligand may be involved.

These dual labeling studies reinforce the dynamic nature of band 3 degradation in the IE membrane, and reveal that multiple neoantigens may be present at different locations within a single membrane. As well, time course studies indicate that there is a temporal order in band 3 neoantigen generation.

PfEMP1 was the other IE ligand examined. Antibodies to this protein first detected its presence in infected cells 8 hours prior to binding. At this time, a ring of intense immunostaining with a small, single unstained region was observed (Figure 16A). This pattern of fluorescence is consistent with the distribution of parasite cytoplasm, and indicates that large amounts of PfEMP1 are synthesized by the late ring parasites.

Four hours prior to the onset of cytoadherence, the parasites are still in the late ring stage. However, the scattered foci of green fluorescence observed in topical views suggest that the protein is no longer evenly distributed in the parasite cytoplasm (Figure 16C). Z-scan analysis confirmed that all PfEMP1 was still confined within the IE membrane since the green fluorescence is contained interior to the area of red Sin-1 polyclonal staining (Figure 16D). It is possible that the scattered foci represent vesicles of PfEMP1 protein either

contained within the parasitophorous vacuole or in transit through the erythrocyte cytoplasm (Lingelbach, 1997).

Z-scan analysis of thin smears prepared at the time of the onset of binding revealed superimposable green and red fluorescence. This indicates that PfEMP1 is exclusively surface expressed at the onset of binding (Figure 16F). Live staining studies also confirmed the surface presence of PfEMP1 at this time (Figure 17).

Thus, temporal binding studies indicate that both PfEMP1 and the band 3 neoantigen recognized by the mAb 1C4 are surface expressed coincident with the onset of binding. Therefore each might act as a functional IE ligand for CD36 or ICAM1. Inhibition studies have revealed that antibodies to each potential ligand significantly reduce binding to CD36, suggesting that both may play a role in CD36 cytoadherence (Baruch *et al.*, 1995; Crandall and Sherman, 1994a). To determine if both proteins are coincidentally expressed on a single IE, dual staining studies were conducted. Indeed, red and green fluorescence was observed on individual cells indicating coexpression (Figure 13C). However, the pattern of surface fluorescence was distinct for each protein denoting different distribution in the IE membrane. Since PfEMP1 has definitively been localized to >50% of knob protrusions of a single IE, (Baruch *et al.*, 1995), this may indicate that the 1C4 neoantigen is present elsewhere on the IE surface, or that it is present in knobs devoid of PfEMP1.

The third specific objective of these studies was to examine the temporal expression of PfEMP1-encoding *var* genes (Figure 10). As predicted by the IFA results, significant levels of *var* gene transcripts were detected in IE 8 and 4 hours prior to binding (Figure 12, Lanes 4 and 5). However, at all other time points, transcripts were almost undetectable. This

suggests that *var* gene transcription is controlled in a stage-specific manner similar to other parasite proteins (Fox *et al.*, 1994; Rojas *et al.*, 1995; Vernot-Hernandez *et al.*, 1984).

The late ring to early trophozoite switch corresponds to a time of burst in gene expression (Vernot-Hernandez *et al.*, 1984). It is possible that the expression of multiple temporally or functionally related proteins is coordinated in a cistron or controlled by a temporally restricted transcription factor(s). Indeed, transcription of another the knob associated protein, KHARP, has been shown to occur at 9 to 21 hours post-invasion (Vernot-Hernandez *et al.*, 1984). This type of transcriptional control might explain why *var* genes continue to be expressed in *in vitro* cultures in the absence of immune selective pressure.

Difficult to reconcile with the temporal expression of *var* genes is the reappearance of PfEMP1 on the IE surface of trypsin treated erythrocytes (Leech *et al.*, 1984). IFA studies do not support cytoplasmic synthesis or storage of this protein subsequent to initial surface expression, and thus suggest *de novo* transcription. How PfEMP1 degradation on the IE surface initiates this transcription and how such mechanisms could be physiologically relevant are unclear.

In addition to regulation of transcription, regulation of *var* gene expression must involve other levels of control. For example, it is evident from Figures 4 and 5 that only a small subset of the *var* gene repertoire is expressed in culture at a given time, and that this subset changes with time. Thus mechanisms to control which *var* genes are expressed may also exist. The continued cycling of *var* genes in culture, in the absence of immunologic selective pressure may be linked to these control mechanisms.

Above and beyond transcriptional control, there must also exist mechanisms controlling the PfEMP1 protein. PfEMP1 accumulates in the cytoplasm of late ring stage

organisms, but is not surface expressed on the IE membrane until the trophozoite stage. It is likely that the delay in surface presence is related to packaging and transport. Parasite derived proteins destined for the IE cell surface must pass through the parasite cytoplasm, the parasite membrane, the parasitophorous vacuole (PV) membrane, the IE cytoplasm and finally insert in the erythrocyte plasma membrane. Although various hypotheses have been presented concerning these matters including the one (Howard *et al.*, 1987) and two step models of transport across the PV membrane, (Haldar, 1996) and the existence of a parasitophorous duct or a tubovesicular membrane or network in the erythrocyte cytoplasm (Pouvelle *et al.*, 1991; Elford *et al*, 1997), the synthesis and transport of parasite proteins in IEs remain poorly understood (Lingelbach, 1997).

Pulse chase experiments have revealed that KAHRP, which similarly is transcribed during the late ring stage, does not become surface expressed for 6 hours following the onset of its transcription (Vernot-Hernandez *et al.*, 1984). It has been suggested that the KHARP protein may interact with the ATS of PfEMP1 in knob structures and thus may play a role in the trafficking of PfEMP1 (Pologe *et al.*, 1987; Crabb *et al.*, 1997).

Thus, transcription, translation, downstream packaging, interaction with KHARP and transport may all influence the surface presence of PfEMP1.

At the time of binding, both PfEMP1 and 1C4 are present on the IE cell surface and therefore can be considered putative cytoadherence ligands. Why would the parasite maintain two individual cytoadherence ligands? Theoretically, sequestration offers the parasite a survival and replication advantage (Howard and Gilladoga, 1989). Multiple IE ligands that bind different endothelial receptors have been identified, including Sequestrin which is also hypothesized to bind CD36 (Ockenhouse *et al.*, 1991)(Chaiyaroj *et al.*, 1994a)(Chaiyaroj *et*

al, 1994b). In addition PfEMP1 has been shown to bind multiple receptors including CD36, ICAM-1 and TSP, and it is estimated that *var* genes comprise 2-6% of the haploid genome (Baruch *et al.*, 1996)(Su *et al.*, 1995). Thus the theoretic advantage of sequestration is substantiated in the adherence characteristics of the parasite.

How could both molecules be involved in IE cytoadherence? The first possibility is that they could act cooperatively to form the functional ligand. Although knobless parasites can adhere to C32 melanoma cells in static adherence assays, (Biggs *et al.*, 1990), KHARP knock-out parasites cannot adhere under physiological flow conditions (Crabb *et al.*, 1997). Perhaps both PfEMP1 and the 1C4 band 3 neoantigen are present in the knob protrusions and interact to form a conformationally relevant ligand. Conflicting with this model are the IFA data: dual labeling demonstrates that the two proteins have distinct distributions on the IE surface. It is conceivable, however, that this staining is artifactual, and if the initial anti-PfEMP1 antibody used sterically hinders neighbouring 1C4 molecules so that second primary antibody used could not access these sites. Still, the distribution of immunostaining with the individual antibodies reveals very distinct staining patterns. Surface staining for 1C4 has a non-uniform, punctate distribution over the IE surface, whereas PfEMP1 is more diffusely dispersed. Thus, these data do not support the hypothesis that these two molecules cooperate to form a functional ligand.

The second possibility is that the two molecules act as independent, redundant ligands; PfEMP1 mediating adherence to both CD36 and ICAM-1, and the 1C4 neoantigen binding only CD36. Since CD36 appears to be the major *in vivo* receptor (Ockenhouse et al., 1991)(Udomsangpetch et al., 1996)(Newbold et al., 1997), the presence of multiple ligands on the IE surface that could interact with this molecule would increase the likelihood that

parasites would sequester. Additionally, it is possible that alternative CD36 ligands might be necessary if certain PfEMP1 variants interacted less avidly with this major receptor. Despite the plausibility of these arguments, recent studies have demonstrated that peptides based on band 3 neoantigens do not bind CD36 directly and therefore this possibility is negated (Lucas and Sherman, 1998).

A third possibility is that PfEMP1 and the 1C4 neoantigen act independently, binding to different receptors. Recent studies have demonstrated that peptides based on band 3 neoantigens interact with TSP rather than CD36, thus supporting this possibility (Lucas and Sherman, 1998). However, it is difficult to reconcile this with the coincident appearance of these two molecules on the IE surface found in these studies. One explanation might be that the insertion of PfEMP1 in the IE membrane creates the 1C4 neoantigen by the mechanical disruption of the native band 3 molecule. Another possibility is that the protease responsible for the creation of the 1C4 neoantigen could be of parasite origin and that its expression is temporally linked to that of the active *var* genes. Alternatively, the 1C4 neoantigen and PfEMP1 surface expression might not be completely coincident.

Gardner and colleagues found that binding to TSP began 2 hours prior to binding to purified CD36 (Gardner *et al.*, 1996). If this adherence is mediated by band 3 neoantigens then it would follow that they should be present on the cell surface 2 hours prior to the onset of binding to CD36. Since the sampling interval in these studies was 4 hours, the resolution is limited, and this event might have been missed. Thus the possibility that PfEMP1 and the 1C4 neoantigen act independently binding to different receptors is plausible.

The last possibility is that one or both of these molecules do not function as a ligand *in vivo*.

Given the importance of cytoadherence to the virulence of *P. falciparum*, it is likely that both PfEMP1 and the 1C4 neoantigen are functional ligands. Although our studies do not negate involvement of both ligands in CD36 binding, studies by Lucas and Sheman indicate that band 3 neoantigens do not interact directly with this receptor, rather they bind TSP. Thus, it is likely that that PfEMP1 and the 1C4 neoantigen act independently, binding to different receptors. This would support the leukocyte recruitment model of cytoadherence and promote effective *in vivo* sequestration.

In summary, the ability to adhere to the biologically relevant receptors CD36 and ICAM-1 is an off/on event closely linked to parasite developmental stage. Within the resolution of the experimental design the onset of binding to both receptors appeared to be coincident and permanent. Additionally, adherence to ICAM-1 was found to be more sensitive to shear stress suggesting that this receptor may mediate rolling rather than static adherence *in vivo*, in accordance with the leukocyte recruitment based model of cerebral sequestration (Cooke *et al.*, 1994; Udomsangpetch *et al.*, 1997).

Four proposed ligands were examined, PfEMP1 and three band 3 neoantigens. The 3H3 and 1F4 neoantigens are unlikely candidates for CD36 or ICAM-1 ligands since their cell surface presence preceded the onset of binding. The 1C4 epitope however consistently appeared on the IE surface coincident with the onset of binding, and is therefore temporally relevant as a potential ligand. These studies also highlighted the dynamic nature of band 3 degradation in the IE membrane, demonstrated the existence of multiple band 3 neoantigens in a single IE and revealed that there is a sequential order in band 3 neoantigen generation.

PfEMP1 was demonstrated to be synthesized intracellularly during the late ring stage and then transported to the IE membrane. Both live staining and confocal microscopy

confirmed the presence of this molecule in the red cell membrane of cytoadherent trophozoites. Additionally, *var* gene transcription was shown to be stage specific with pronounced upregulation 8 hours prior to surface expression. Additional levels of transcriptional control are suggested by the sequential expression of a small subset of *var* genes *in vitro* culture, and downstream trafficking control is consistent with the long lag period between protein synthesis and IE membrane localization.

In conclusion, these time course studies indicate that the ability of IEs to bind both CD36 and ICAM-1 appears to be an off/on event which could be mediated by PfEMP1 or the band 3 neoantigen 1C4 or both. However, due to the limited resolution of these experiments the possibility that the band 3 neoantigen appears on the IE surface slightly earlier than PfEMP1 and mediates adherence via the TSP molecule could not be ruled out. This information provides insight into the molecular events involved in cytoadherence, and may eventually help elucidate the pathophysiology of cerebral malaria.

FUTURE STUDIES

These studies have suggested new avenues of research that might contribute to our understanding of the roles of the receptors and ligands in cytoadherence and sequestration.

With respect to band 3 degradation in the IE membrane, these studies suggest that there is a specific temporal order in which epitopes are created. This may be due to sequential, conformation-dependent breakdown of the protein by a single protease, or the temporal involvement of several proteases. This might be explored by examining the proteolytic activities of IE in time course studies similar to those conducted in these studies.

Another avenue that would be interesting to explore is the ultrastructural location of the 1C4 neoantigen. It has been suggested that band 3 neoantigens are present in IE knobs, (Winograd *et al.*, 1989), although this has not been definitively demonstrated for the 1C4 neoantigen. If immunogold staining revealed that this was the case, then multiple cross sections of a single knob could be stained to determine if the PfEMP1 and 1C4 ligands colocalize. If they do, this would promote the hypothesis of an interaction of the two molecules to form a functional ligand. Localization of the 1C4 neoantigen would also provide some insight into the hypothesis mentioned earlier, that knob formation creates the 1C4 neoantigen by mechanical disruption of the band 3 molecule.

The control of *var* gene expression is another potential area for future studies. Based on the pattern of transcript production seen in these experiments it was suggested that transcriptional control might involve upregulation by some activating protein, or suppression by the abbreviated 3' transcript species. An experimental design similar to that used in this work could be used to examine the latter of these two possibilities. By performing quantitative RT-PCR or northern blot analysis which targets both the 5' and 3' ends of the

var gene transcripts, one could determine the relative abundance of full length and truncated transcripts, and elucidate whether the latter could play an inhibitory role.

If this does not appear to be the case, understanding of the temporal onset of transcription might aid in the search for a temporally controlled transcriptional activator protein. Moreover, experiments designed to artificially induce the upregulation of *var* gene transcription, such as IE trypsinization might be designed. This would reveal whether or not additional temporally restricted factors are involved in *var* gene transcription and also might aid in the search for an activator protein.

On the receptor side, it would be interesting to determine if the identity of the "unknown receptor" to which ItG parasites could be selected to bind is CSA. This could be examined by inhibiting binding with soluble CSA or chondroitinase, or by examining binding to purified potential receptor adhered to plastic.

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