

# Studies on the glycoprotein modification in erythrocyte membrane during experimental cerebral malaria

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## Abstract

*Plasmodium berghei ANKA (Pb ANKA)* is a lethal strain of malaria that causes experimental cerebral malaria (ECM) in rodent models. Pathology of the disease is associated with the sequestration of the infected rbc (irbc) in the micro vessels of brain. In the present study, we analyzed the nature of the glycoprotein modification occurring in irbc membrane during erythrocytic stages of *Pb ANKA* infection. Titration of naturally occurring glycoproteins with concanavalin A (Con A) and wheat germ agglutinin (WGA) lectins revealed an enhanced lectin binding ability for the irbc membrane preparations. Partial characterization of the Con A specific determinants ( $\alpha$ -D-methyl mannoside specificity) by lectin affinity chromatography followed by 2D electrophoresis and WGA specific determinants (sialic acid specificity) by Western analysis revealed the association of novel lectin specific determinants in irbc membrane. To correlate the biochemical changes with the morphological changes, SEM of irbc, and TEM of sequestered irbc were performed. These ultra structural studies revealed variable and irregular surface protrusions and deep surface indentations on irbc. These observations implicate that altered glycoprotein profiles may lead to cytoarchitectural changes in irbc membrane and such changes may be essential to establish contact with the host endothelial cells. These observations may be central to the microvascular sequestration and pathology of ECM.

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*Index Descriptors and Abbreviations:* Cerebral malaria; rbc membrane; Lectins; Microvascular sequestration

## 1. Introduction

*Plasmodium falciparum (Pf)* malaria is the most important parasitic disease infecting the central nervous system (CNS) of humans worldwide. It infects 300–500 million people each year and causes over one million deaths (Gitau and Newton, 2005). The pathology of the disease is associated with the ability of irbc to adhere to the endothelial cells of the microvasculature of numerous deep tissues (Ho and White, 1999; Sherman et al., 2003). Termed as sequestration, this phenomenon facilitates the multiplication of parasites and avoids splenic clearance of the parasites (Chotivanich et al., 2002; Engwerda et al., 2005). Cytokines

like TNF- $\alpha$  play an important role in the pathogenesis of CM (Grau et al., 1989; Kwiatkowski, 1990) and are known to up regulate a number of adhesion molecules, including ICAM-1/CD54 (Hviid et al., 1993), CD36, and thrombospondin (Newbold et al., 1997) on vascular endothelia of major organs of the body including brain. Ligation of novel antigenic determinants appearing on the surface of irbc to any of these endothelial receptors is hypothesized to alter the integrity of the tight junctions of the brain micro vessels. Breakdown of blood-brain-barrier (BBB) causing leakage of inflammatory mediators into CNS that eventually affect neuronal function has been proposed as a possibly important mechanism of CM malaria pathology (Adams et al., 2002).

Despite the increasing knowledge of a range of molecules involved in binding of irbc to endothelial cells, the specific interaction that lead to pathology are yet to be

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established. In the present study, we used a lectin based immunological approach to study the surface properties of irbc in experimental cerebral malaria model induced by *Plasmodium berghei ANKA*. Titration of naturally occurring glycoproteins using Con A and WGA lectins revealed an enhanced binding ability of irbc to these lectins. Concomitant with these observations we also demonstrate the presence of novel antigenic determinants or cryptic residues specific to these lectins in the irbc membrane preparations. Altered glycoprotein profiles may lead to cytoarchitectural changes in irbc membrane and such changes may be essential to establish contact with the host endothelial cells, as shown by our EM studies. Such interactions may be crucial to sequestration and pathology of cerebral malaria.

## 2. Materials and methods

### 2.1. *In vivo* propagation of *Pb ANKA* parasites

*Plasmodium berghei ANKA* strain was obtained from Malaria Research Center (MRC), Delhi. The parasites were stored as frozen stocks at  $-80^{\circ}\text{C}$ . Freshly thawed malaria parasites were passaged once through mice, and all mice were infected at 3–4 weeks of age by intraperitoneal injection of  $1 \times 10^4$  parasitized erythrocytes (Hearn et al., 2000). The parasitaemia was monitored by geimsa stained blood smears made from caudal vein puncture. Mice had an infection of around 1% by day 2 post infection. The parasitaemia progressively increased there after, reaching 15–20% between day 6 and 8 and infection was lethal by day 10.

### 2.2. Preparation of rbc membrane

Infected blood was obtained from cardiac puncture in an anticoagulant heparin solution (Sigma). The infected blood was washed 3–4 times in 5 mM PBS, pH 7.4. The cells were resuspended in fresh 5 mM PBS and passed through CF-11 cellulose column (Sigma) to deplete the leukocytes and the platelets from the blood. The process was repeated 3–4 times to obtain a pure preparation of rbc. Erythrocyte membrane ghost were generated by hypotonic lysis (Dodge et al., 1963) in a buffer containing protease inhibitor cocktail (4-(2-aminoethyl) benzene sulfonyl fluoride (AEBSF), E-64, bestatin, leupeptin, aprotinin, and sodium EDTA) (Sigma). Lysing was done under constant stirring conditions at  $4^{\circ}\text{C}$  to facilitate the release of parasites by mechanical rupturing of the irbc. The lysed cells were initially spun at 150 g for 1 min to pellet the parasites and unlysed cells. The supernatant was centrifuged at 36,000 rpm to pellet down the infected membranes. The membranes were washed for 3–4 times in 5 mM PBS (pH 7.4) and solubilised in 1% Triton X-100 detergent. Control rbc membranes were similarly prepared from blood obtained from uninfected mice. Protein estimation for rbc membrane was done by the method of Lowry et al. (1951).

### 2.3. Lectin immunization and preparation of IgG

Five hundred micrograms of both Con A and WGA lectins (Bangaloregenei, India) were emulsified with complete (first booster) and incomplete (second booster) Freund's adjuvant (Alves et al., 2000). Rabbits were ear bled 2 weeks after final immunization. The antisera from several bleeds were pooled and IgG fractions were affinity purified on a protein G column (Bangaloregenei, India). The purity of the antibodies was evaluated on a 10% SDS-PAGE gel. Purified antibodies were used for lectin based immunological titrations.

### 2.4. Titration of naturally occurring glycoproteins of irbc against Con A and WGA lectins

Titration of naturally occurring glycoproteins with specific affinity for Con A and WGA lectins was performed by coating  $5 \mu\text{g}/\text{well}$  of control and irbc membrane (solubilised in 1% Triton X-100) to ELISA plates. Coating was done overnight at  $4^{\circ}\text{C}$ . The plates were subsequently blocked with 1% oxidized BSA for 2 h at room temperature. The plates were rinsed twice with PBS (pH 7.4) and once with 0.05% PBST. The wells were replaced with a solution containing  $10 \mu\text{g}/\text{ml}$  of either Con A or WGA made in 10 mM Tris buffer, pH 7.4, containing 1 mM of each  $\text{MnCl}_2$ ,  $\text{CaCl}_2$ , and  $\text{MgCl}_2$ . The concentration of lectin was optimized and fixed to  $10 \mu\text{g}/\text{ml}$  after performing a series of titrations using varying range of lectin concentrations (data not shown). After 2 h treatment with respective lectins, the plates were washed 4 times with PBS, followed by incubation with affinity purified IgG (concentration  $1 \mu\text{g}/\text{ml}$ ) specific for Con A or WGA, respectively, for additional 1 h. This was followed by incubation with anti-rabbit secondary antibody conjugated to horse radish peroxidase (Sigma). The lectin immunoreactivity was quantified by adding *o*-dianisidine ( $10 \text{ mM } o\text{-dianisidine}$  in ethanol +  $3 \mu\text{l}$  of  $\text{H}_2\text{O}_2$ ) (Sigma) that developed a chromogenic reaction. After developing optimal color, the plates were read at 407 nm. The specificity of interaction of Con A and WGA with erythrocyte membrane glycoproteins was established by complete inhibition of lectin binding in the presence of  $\alpha\text{-D-methyl mannoside}$  and sialic acid, respectively, when a concentration of 10 mM of these respective sugars were mixed along with the lectin. To quantify the lectin binding, a mean of three optical density values obtained from irbc membrane sample for each lectin was compared with that of the corresponding control by using the Student's *t* test.  $P < 0.05$  was considered significant.

### 2.5. Characterization of the oligosaccharide composition of infected erythrocyte membrane glycoproteins

Binding of glycoproteins to Con A (sugar specificity:  $\alpha\text{-D}$  mannose) was used as a criteria to analyze the composition of the sugar moieties on the oligosaccharide chains of the irbc membrane glycoproteins. The rbc membrane

preparations as described earlier, were diluted in equilibration buffer containing 10 mM Tris, pH 7.4, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, and 1 mM MnCl<sub>2</sub>. The Con A lectin column (Bangaloregenei, India) was equilibrated with same equilibration buffer and samples were applied to the column. Adsorption of the glycoproteins to the column was done for 1 h at 4 °C. The column was thoroughly washed and the bound glycoprotein was eluted with 10 mM  $\alpha$ -D-methyl mannoside. The eluted glycoproteins were extensively dialyzed and concentrated prior to electrophoretic studies.

#### 2.6. Two-dimensional gel electrophoresis of Con A purified glycoprotein

The glycoproteins purified from control and irbc membrane on Con A column were further characterized with regard to their isoelectric points and molecular weights by subjecting them to 2D electrophoresis (O' Farrell, 1974). The Con A purified glycoproteins were solubilised in 9.2% urea, 2% ampholines, and the samples were loaded on iso-electric focusing gel. The iso-electrofocussing was achieved with 2% ampholines of pH 3–10 and 5–8 in the ratio of 1:4. After prefocussing, the gels were run at 620 V/h for 13 h. The gels were removed after run and subjected to equilibration for 15 min. After equilibration, the gels were subjected to second dimension run. Electrophoresis of the second dimension was performed at 15 mA/gel until the dye entered the resolving gel followed by running the gel at 20 mA until the end of the run. The gels were silver stained to visualize the protein spots (Blum et al., 1987).

#### 2.7. Western analysis of WGA specific determinants in irbc membrane

Thirty micrograms of either control or infected erythrocyte membrane were subjected to desialation by enzymatic treatment with 150 mU/ml of *Vibrio cholerae* neuraminidase (EC 3.2.1.18; Sigma) for 2 h at 37 °C with gentle agitation. Incubation of samples in heat inactivated neuraminidase, or none (no neuraminidase treatment) were kept as controls to demonstrate the sialidase activity of enzyme. Following this treatment, the samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970). The gels were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The PVDF membranes were probed with homologous mouse immune sera (1:100 dilution), followed by anti-mouse IgG conjugated to horse radish peroxidase. The blots were developed following exposure to 0.005% H<sub>2</sub>O<sub>2</sub> containing Diaminobenzidine (Sigma). The specificity of antigen recognition by immune sera was confirmed by probing an identical set of membranes either with preimmune sera (1:100 dilution) or none. When revealed with anti-mouse IgG, no specific bands were detectable (data not shown).

#### 2.8. Scanning electron microscopy (SEM)

Cells were fixed in 2% glutaraldehyde (2% in 0.1 M sodium phosphate buffer, pH 6) for 90 min at 4 °C and were washed with decreasing concentration of phosphate buffer followed by dehydration in graded ethanol (from 50%) and finally in absolute ethanol. Dehydrated samples were mounted on a brass stub and were dried at room temperature and sputter coated with gold for 10 min at 1.2 kV. The cell surface architecture was visualized in a JOEL JSM 35 microscope, operated at 25 kV accelerating potential.

#### 2.9. Transmission electron microscopy (TEM)

The infected brain samples were obtained by decapitation of the terminally ill animals that demonstrated several neurological manifestations as described earlier (Kumar and Babu, 2002). The samples were preserved in 10% neutral buffered formalin solution (Sigma) until further processing. When processing resumed, formalin-fixed specimens were extensively washed in PBS, fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, and post fixed with 2% osmium tetroxide for 2 h. The cortex and cerebellar regions were identified with dissection microscope, and 2 × 2 mm sections were cut out of the tissue slices, dehydrated in graded series of ethanol, and embedded in Spur epon. Blocks were trimmed and semithin 0.5  $\mu$ m thick sections were cut with an ultramicrotome, stained with toluidine blue and examined by light microscopy for overall view. Ultra thin 70–90 nm thick sections were then cut, picked up on 200 mesh copper grid, double stained with uranyl acetate and lead citrate, and scanned in JOEL 100 CX electron microscope.

### 3. Results and discussion

#### 3.1. Upregulation of Con A and WGA specific determinants on irbc during malaria infection

Infection of rbc with malaria parasites introduces several changes in the composition of the erythrocyte membrane. In addition to the reorganization of the membrane material during intraerythrocytic growth of malaria parasites (Parker et al., 2004) appearance of novel antigenic determinants may alter profoundly the glycoprotein profiles of the irbc. The neo antigens that appear during infection may perform several functions notably, as ligands for cytoadherence or sequestration that enable, respectively, retention of parasitised rbc in specific organs or help to avoid splenic clearance (Gardner et al., 1996). Evidence for association of irbc in close proximity to the host endothelial cells is clearly established in both the rodents and human CM cases (Hearn et al., 2000; Gitau and Newton, 2005). In the present study, we investigated if the altered antigenic composition of the irbc had different binding abilities to lectins, a class of glycoproteins routinely used as probes to study the surface properties of the rbc. The selection of lectins for

such studies was based on their ability to bind a relatively large number of proteins or sugar residues, which would help in detecting changes in at least few glycoproteins that may be prone to undergo alterations during malaria infection. For this reason we choose Con A and WGA lectins that have broad recognition specificity to  $\alpha$ -D-methyl mannoside and sialic acid, respectively, and focused to study membrane associated protein/glycoproteins changes specific to these lectins. A preliminary immunological titration of the infected vs uninfected erythrocyte membrane using these two lectins revealed an enhanced lectin binding ability of irbc as compared to their control counterparts (Fig. 1). These results suggested an overall modification in the glycoprotein content specific to both Con A and WGA during malaria infection. To further characterize the novel determinants specific to Con A, we subjected Con A affinity purified proteins to 2D gel electrophoresis (Fig. 2). In addition to a set of proteins common to both control and

infected erythrocyte membrane, we identified few novel proteins and defined them with regard to their molecular weights and isoelectric point as follows: A, 97 kDa and pI 4.8; B, 66 kDa and pI 4.8; C, 50 kDa and pI 5.6; D, 55 kDa and pI 6; and E, 29 kDa and pI 5.2. Similar characterization of the WGA specific determinants was done with respect to their molecular weight (Fig. 3). The fact that neuraminidase treatment removes the sialic acid determinants from irbc and renders these samples insensitive to recognition by homologous immune sera was used as a criterion to identify the WGA specific determinants. This strategy enabled us to identify antigens of molecular weight 97, 66, and 50 kDa on Western blots specific to irbc membrane samples. The newly appearing lectin specific molecules in the irbc membrane may serve different functions notably cytoadherence and sequestration. For example, binding of human irbc to host adhesion molecule, ICAM-1 (Kaul et al., 1998; Newbold et al., 1997) occurs via receptors on

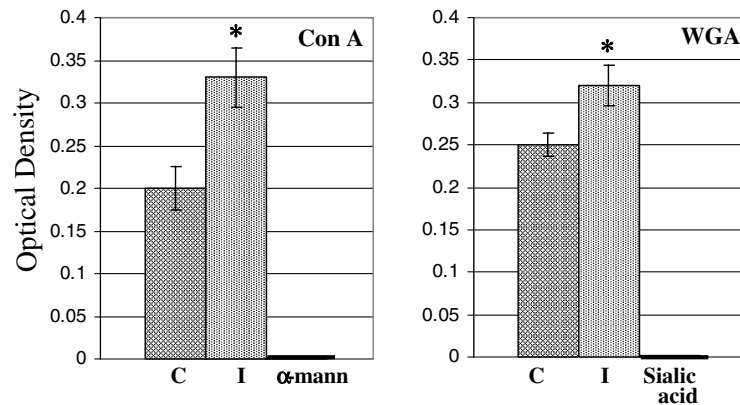


Fig. 1. Titration of naturally occurring glycoproteins from control and infected erythrocyte membrane were performed by using an enzyme-linked immuno lectin assay as described in Section 2. In brief, 5  $\mu$ g/ml of control (C) or infected (I) erythrocyte membrane was coated to ELISA plates followed by treatment with either Con A or WGA lectin at concentration of 10  $\mu$ g/ml. The lectin binding was quantified by using affinity purified antibodies specific for Con A or WGA. The immunoreactivity of the lectin and antibody reaction was detected by using anti-rabbit secondary antibody conjugated to horse radish peroxidase and revealed by peroxidase substrate, *o*-dianisidine, and the optical density was recorded at 407 nm. The specificity of interaction of Con A and WGA with erythrocyte membrane glycoproteins was established by complete inhibition of lectin binding in the presence of  $\alpha$ -D-methyl mannoside ( $\alpha$ -mann) or sialic acid, respectively, when a concentration of 10 mM of these respective sugars were mixed along with the lectin. To quantify the lectin binding, a mean of three values (optical density) obtained from irbc membrane samples for each lectin was compared with that of the corresponding control by using the student's *t* test. \**P* < 0.05 was considered significant.

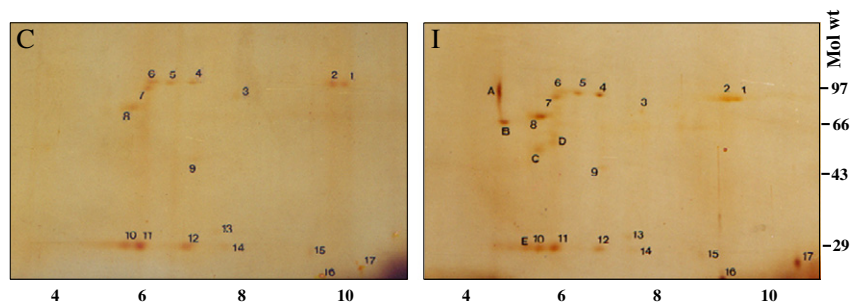


Fig. 2. Two-dimensional analysis of Con A affinity purified proteins from control (C) and infected (I) erythrocyte membrane was performed as described in Section 2. The Con A affinity purified glycoproteins were solubilised in 9.2% urea, 2% ampholines, and the samples were loaded on to iso-electric focusing gel. The iso-electric focusing was achieved with ampholines of pH 3–10 and 5–8 in the ratio of 1:4. Following first dimension electrophoresis, the gels were subjected to second dimension SDS-PAGE. The resolved proteins were analyzed by silver staining. Numbered 1–17 were protein spots common to both samples. Indicated in alphabets (A–E) were proteins specific to infected erythrocyte membrane. Their respective molecular weight and pI are described in the text. The pI gradient (4–10) is indicated at the bottom of the gel. Molecular weight marker standards (kDa) are indicated on the right.

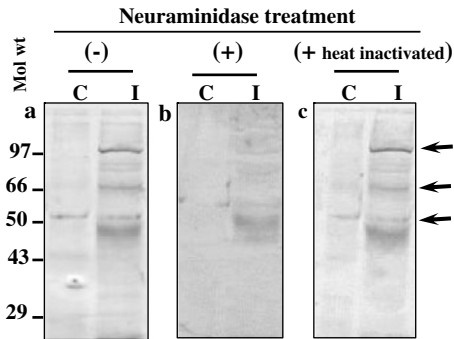


Fig. 3. Western blot analysis for detecting the sialic acid determinants in infected erythrocyte membrane. Three sets of Control (C) and Infected (I) rbc membrane preparations (30  $\mu$ g each) were either subjected to (a) no neuraminidase treatment (–), or (b) neuraminidase treatment (150 mU/ml) (+) or (c) heat inactivated neuraminidase treatment (+ heat inactivated) as described in materials and methods. Following enzymatic treatment, the samples were analyzed on Western blots probed with immune sera obtained from infected mice. The arrow marks indicate sialic acid determinants of molecular weight 97, 66, and 50 kD in both (a) and (c). Loss of immunoreactivity towards the aforementioned bands upon neuraminidase treatment (b) confirmed the sialic acid specificity of these antigens. Molecular weight marker standards (kDa) are indicated on the left.

parasitized rbc surface that leads to sequestration typical of cerebral malaria (Aikawa et al., 1990). The receptors have been identified as PfEMP1 (Pasloske and Howard, 1994). In addition to the parasite specific antigens, changes in the erythrocyte glycoprotein composition may also change the phenotype of the cells with regard to their rosette formations. It has been suggested that rosette formation between uninfected and infected erythrocytes (Rowe et al., 1995) and cytoadherence of the rosettes to the endothelium via CD36 or CD31 (Fernandez et al., 1998) may have important bearing on sequestration and outcome of human CM. A lectin based approach to preselect a subset of these antigenic determinants appearing in rodent malaria infection should be a first logical step to narrow our search to relevant determinants crucial to pathogenesis.

### 3.2. Homologous mouse immune sera recognize distinct antigens in control and irbc membrane

Modification of the host erythrocyte cytoskeletal composition may also expose certain cryptic residues that additionally favor cytoadherence or sequestration. To test this hypothesis, we used homologous mouse immune sera against control and infected rbc membrane preparation by a Western blot analysis (Fig. 4). Our results showed that immune sera recognized similar bands as described above with sialic acid specificity, in addition to a 29 kDa band in the irbc membrane. In addition, the immune sera also recognized some high molecular weight proteins (in the range of 70–80 kDa approximately) in the control rbc membrane preparation (Fig. 4). The immunoreactivity of immune sera for high molecular weight bands in control membrane sample and several low molecular weight bands in the infected membrane could suggest the degradation of native proteins

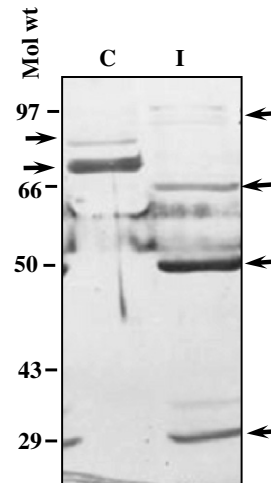


Fig. 4. Western analysis of control (C) and infected (I) erythrocyte membrane proteins using homologous immune sera to detect parasite induced changes. Marked with arrows in control and infected lanes are several protein bands recognized by immune sera. Molecular weight marker standards (kDa) are indicated on the left.

during infection. Consistent with our observations in the rodent models, an example of such membrane modification has been recently described in *Pf* infected erythrocytes (Winograd et al., 2005) where anti-peptide antibodies generated against amino acid sequence YETFSKLIKIFQDH of human band-3, which was previously identified as mediating adhesion of irbc to CD36, recognized infected *Pf* rbc. In addition, sera obtained from malaria endemic area contained immunoglobulins specific for this region of band-3. In uninfected rbc, band-3 region was cryptic and its exposure on *Pf* infected rbc required clustering of band protein. This observation provides a direct evidence for a parasite induced modification of the erythrocyte membrane that promotes adhesion and induces antigenic changes in *Pf* infected rbc.

### 3.3. Cytoarchitectural changes of irbc

To correlate the antigenic changes of the irbc membrane with their ability to get sequestered in brain micro vessels, we analyzed at the ultra structural level, the proximity of irbc with the host cells. Such studies are important to address the relevance irbc in activation or disruption of BBB. TEM (Figs. 5A and B) and SEM of isolated irbc (Fig. 5C) showed variable and irregular surface protrusions and deep surface indentations. Membranous protrusion of distinct regions of irbc could possibly help in establishing contact with host endothelial cells or microglial cells. Association of irbc with the brain resident macrophages may provide them with a signal to produce inflammatory cytokines like TNF, interleukin-1, and gamma interferon that upregulate a number of adhesion molecules including ICAM-1/CD54 (Hviid et al., 1993), CD36, and thrombospondin (Newbold et al., 1997) on vascular endothelia of major organs including brain. A recent in vitro study (Treatanapiboon et al., 2005) demonstrated that membrane

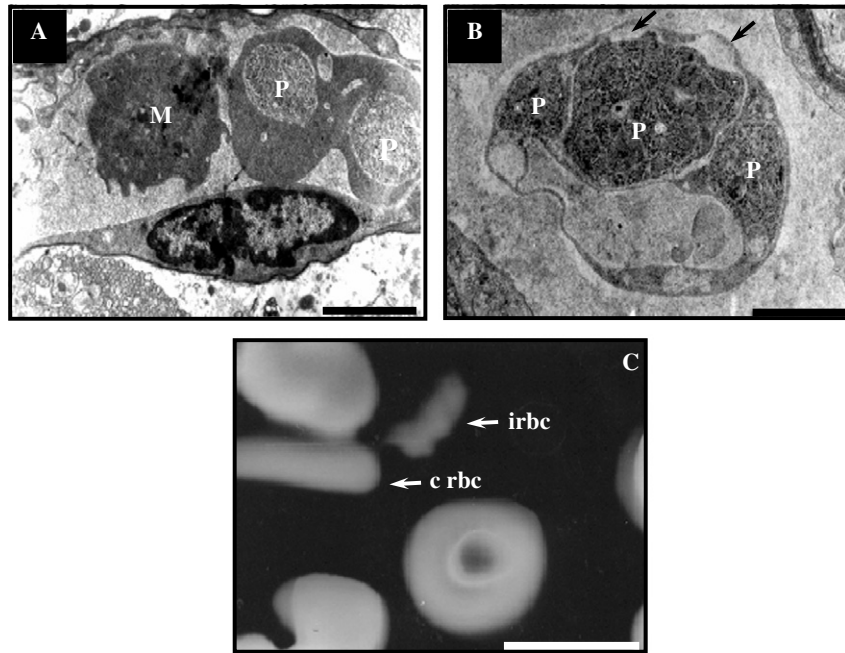


Fig. 5. TEM showing ultrastructural details of capillary from infected brain. Shown in (A) is a capillary from the cerebellar parenchyma occluded by monocytes (M) and infected rbc (irbc) harboring two parasites (P). Bar, 3  $\mu$ m. Shown in (B) is a single irbc harboring more than one parasite. Indicated with arrow marks are distinct membranous projections in vicinity to vascular endothelia. Bar, 3  $\mu$ m. Shown in (C) is SEM of rbc. Indicated with arrow is an irbc showing cytoarchitectural changes with distinct membrane protrusions, in comparison to normal discoid structure of control rbc (c rbc). Bar, 7  $\mu$ m.

associated malaria antigens are capable of stimulating human peripheral blood monocytes to secrete tumor necrosis factor alpha. In a co-cultivation with brain endothelial cell model, malaria activated human PMBC stimulated the expression of E-selectin and ICAM-1 on the porcine brain capillary endothelial cells (PBCEC). Using electric cell-substrate impedance sensing, these studies documented a significant decrease of the endothelial barrier function within 4 h incubation with malaria activated PMBC. Concomitant with these changes, immunocytochemical studies showed the disruption of tight junction complexes.

Several signaling molecules specific to tight junctions are being implicated during cerebral malaria (Adams et al., 2002). The counterparts for activation of host endothelial cells have been described as parasite antigens in various instances. Using a rodent model of cerebral malaria, we demonstrate an altered topology of irbc as evinced by their differential binding to lectins. We hypothesize that the altered binding properties confer on irbc the ability to adhere and signal the host endothelial cells and other immune cells residing in the brain. Junctional proteins between endothelial cells are known to regulate cell signaling, gene transcription, and paracellular permeability (Staddon et al., 1995; Staddon and Rubin, 1996). Adhesion specific receptor-mediated events, such as ICAM-1 binding to host endothelial cells or non-adhesion dependent circulating mediators could activate signaling pathways through junctional proteins. This would allow the leakage of plasma proteins and inflammatory mediator into the perivascular spaces, causing activation of astrocytes and microglia during CM. The rationale for such hypothesis is based on the

observation that LFA mediated lymphocyte adhesion to ICAM-1 (Adams et al., 2002) leads to activation of focal adhesion kinase (FAK), alterations of cellular cytoskeletal components, and possibly to the increased junctional permeability. Our current studies documenting monocytes in vicinity to irbc and brain endothelial cells (Fig. 5A) are a clear indication of a local inflammatory response during ECM. Further, our own published data demonstrate the activation of apoptosis like events characterized by mitochondria dysfunctions (Kumar and Babu, 2002) and activation of NF- $\kappa$  B (Kumar et al., 2003) during ECM. An emerging theme from the biochemical, ultrastructural, and cell signaling studies clearly implicate a complex network of interaction at the interface of irbc and host cells. A more detailed understanding of the association between these events shall greatly assist in development of effective chemotherapeutic interventions.

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