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PLASMODIUM COATNEYI-INFECTED RHESUS MONKEYS: A PRIMATE MODEL FOR HUMAN CEREBRAL MALARIA

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Although several animal models for human cerebral malaria have been proposed in the past, none have shown pathological findings that are similar to those seen in humans. In order to develop an animal model for human cerebral malaria, we studied the pathology of brains of Plasmodium coatneyi *(primate malaria parasite)-infected rhesus monkeys. Our study demonstrated parasitized erythrocyte (PRBC) sequestration and cytoadherence of knobs on PRBC to endothelial cells in cerebral microvessels of these monkeys. This is similar to the findings seen in human cerebral malaria. Cerebral microvessels with sequestered PRBC were shown by immunohistochemistry to possess CD36, TSP and ICAM-1. These proteins were not evident in cerebral microvessels of uninfected control monkeys. Our study indicates, for the first time, that rhesus monkeys infected with* P. coatneyi *can be used as a primate model to study human cerebral malaria.*

Key words: *Plasmodium coatneyi* – human cerebral malaria – animal model – pathology – rhesus monkeys – brain

Human cerebral malaria is a pernicious manifestation of infection with *Plasmodium falciparum*. The blockage of cerebral vessels has been considereḑ to be the major factor in the pathogenesis of human cerebral malaria based on clinico-pathological studies (Aikawa 1988; Aikawa et al., 1990; Riganti et al., 1990; Pongponratn et al., 1991).

Electron microscopy revealed multiple electron dense knobs protruding from the membrane of PRBC seen in the cerebral microvessels of cerebral malaria patients (Aikawa, 1988; Aikawa et al., 1990; Riganti et al., 1990). These knobs attach via a parasite ligand to receptors on endothelial cells, resulting in the blockage of cerebral microvessels. Recently, several investigators reported that host cell molecules such as CD36 (Barnwell et al., 1989), thrombospondin (Roberts et al., 1985) and intercellular adhesion molecule-1 (ICAM-1) (Berendt et al., 1989) may function as the endothelial cell surface receptors for PRBC. However, there is little information as to whether these molecules actually play a role in cytoadherence of PRBC *in vivo*.

In the past, *P. yoelii* - and *P. berghei* - infected rodents have been reported to develop cerebral malaria and have been used as animal models for human cerebral malaria (Grau et al., 1990). However, cerebral malaria in rodents does not appear to be the same as human cerebral malaria, since erythrocytes infected with rodent malarial parasites do not form knobs nor adhere to the endothelial cells (Aikawa, 1988). No blockage of cerebral microvessels by PRBC can be seen in these animals. (Aikawa, 1988). Instead, in rodent malarias, monocytes accumulate in cerebral

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vessels. This phenomenon is rarely seen in human cerebral malaria.

Plasmodium coatneyi produces knobs on the membrane of PRBC (Rudzinska & Trager, 1968) and these PRBC appear to sequester in the vasculature of infected rhesus monkeys (Desowitz et al., 1969). There have been no reports, however, indicating whether cytoadherence of PRBC to endothelial cells of cerebral microvessels occurs *in vivo.* Therefore, we studied the pathology of the central nervous system (CNS) of rhesus monkeys infected with *P. coatneyi* in order to determine whether cytoadherence of PRBC to endothelial cells is a consistent feature of infections with this primate parasite and, if so, whether putative receptors for cytoadherence in human cerebral microvessels such as CD36, TSP and ICAM-1 are also present.

MATERIALS AND METHODS

Infection of rhesus monkeys – Five non-splenectomized adult rhesus monkeys, (*Maccaca mulatta*) were selected from a closed primate colony at the Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand. Three clinically normal monkeys (AF-B464, AF-G425 and AF-B298) were inoculated via the femoral vein blood-stage parasites of the type strain (Eyles, 1963) of *P. coatneyi.* Tissues from two rhesus monkeys (AF-3927 and AF-233) which were euthanized for other purposes were used as controls. Two splenectomized adult rhesus monkeys (*M. mulatta*) (CDC-3 and CDC-838) with no prior exposure to *P. coatneyi* from the Centers for Disease Control (CDC), Atlanta, Georgia were also inoculated intravenously with the type strain of *P. coatneyi.*

Restraint and examination of monkeys was accomplished after intramuscular injection of Ketamine hydrochloride at 10 to 20 mg./kg. All animals were euthanized with an overdose of intravenous barbiturate. Euthanasia was performed when an animal became severely depressed or developed a static parasitemia. Tissues were taken during a routine necropsy and immersion-fixed for light microscopy, immunohistochemical and electron microscopy evaluation, in 10% neutral buffered formalin, periodate-lysine-paraformaldehyde fixative procedure (McLean & Nakane, 1974) or 2.5% glutaraldehyde in 0.05M phosphate buffer with 4% sucrose, respectively.

Light and electron microscopy – All tissues were examined by light, and electron microscopy. For light microscopy, tissues were embedded in paraffin and sections stained with hematoxylin and eosin. PRBC sequestration was quantified by the examination of 100 cerebral microvessels from each monkey and expressed as the percentage of cerebral microvessels with sequestered PRBC. For electron microscopy, tissues were post-fixed in 1% osmium tetroxide in 0.05M phosphate buffer PO_4 = (pH 7.4) for 1 hour, stained en bloc with 1% aqueous uranyl acetate, dehydrated in ascending concentrations of alcohol and embedded in Epon 812. The sections were cut by a Porter-Blum MT-2 ultramicrotome and were examined by a JEOL 100CX electron microscope.

Immunofluorescent microscopy – For immunohistochemistry, tissues fixed in periodate-lysine-paraformaldehyde fixative were processed for immunofluorescence microscopy (Aikawa et al., 1990). The anti-TSP antibody was a rabbit polyclonal antiserum raised against human platelet-derived TSP that reacts specifically with TSP on Western blotting, immunoprecipitation and ELISA methods (Roberts et al., 1985). An anti-CD36 polyclonal antiserum was produced by immunization of a rabbit with human platelet-derived CD36 that had been purified by gel-filtration and ion-exchange chromatography followed by preparative SDS-polyacrylamide gel electrophoresis and excision of the CD36 band (T. Hasler et al, manuscript in preparation). The animal was immunized repeatedly with the homogenized acrylamide gel slice emulsified in Freund's complete adjuvant and boosted repeatedly with antigen emulsified in Freund's incomplete adjuvant. This anti-CD36 serum was specifically reactive with human CD36 by Western blotting, immunoprecipitation and ELISA methods. To detect ICAM-1 we utilized an anti-ICAM-1 monoclonal antibody, kindly provided by Dr Masashi Matsui and his colleagues, New York Medical College, New York (Matsui et al., 1987). Controls of normal rabbit sera and an irrelevant antibody were employed.

RESULTS

Each of three infected AFRIMS monkeys tolerated *P. coatneyi* infection initially without any behavioral changes. Subsequent signs of illness ranged from partial anorexia in AF-G425 to complete anorexia, total lethargy and inactivity with hemolytic anemia and hypotension

TABLE I

Pheripheral blood parasitemia and percentage of PRBC sequestration in cerebral microvessels at death

	Parasitemia Parasitized cells/mm^3 (% of erythrocytes infected)	Sequestration[a]
AF - B464	257,000 (8.5%)	40
AF - G425	10,193 (0.33%)	35
AF - B298	465,120 (15.5%)	4
CDC - 3	128,000 (4.3%)	29
CDC - 838	560,000 (18.7%)	28

a: sequestration (% of cerebral microvessels with sequestered infected cells).

TABLE II

Parasite stages of PRBC in peripheral blood at the time of death

	Differential parasite count (% of total PRBC)		
	Rings	Trophs.	Schizonts
AF - B464	5	94	1
AF - G425	54	46	0
AF - B298	35	65	0
CDC - 3[a]	36	6	58
CDC - 838[a]	33	15	52

a: monkeys splenectomized before. *P. coatneyi* infection.

in AF-B464 and AF-B298. The two infected CDC splenectomized monkeys, CDC-3 and CDC-838, developed partial anorexia initially and a depressed attitude became evident a few days before euthanasia.

Parasitemia in the five rhesus monkeys infected with *P. coatneyi* ranged from 10,193 parasites/mm^3 to 560,00 parasites/mm^3 at time of death (Table I). Monkeys without splenectomy (AF-B464, AF-G425 and AF-B298) had few schizonts in their peripheral circulation, while splenectomized monkeys (CDC-3 and CDC-838) showed high numbers of schizonts (Table II).

Sequestration of PRBC in cerebral microvessels was evident in all five infected monkeys. Quantitative histopathological examination of 100 random cerebral microvessels revealed various degrees of PRBC sequestration in these monkeys (Table I). Monkey AF-B464 showed PRBC sequestration in 40% of cerebral microvessels, monkey AF-G425 showed PRBC sequestration in 35% of cerebral microvessels and monkey AF-B298 showed PRBC sequestration in only 4% of cerebral microvessels. With splenectomized animals, monkey CDC-3 showed PRBC sequestration in 28% of cerebral microvessels and monkey CDC-838 showed PRBC sequestration in 29% of cerebral microvessels. Representative tissue specimens from various parts of the brain, such as frontal and parietal lobes, midbrain and cerebellum, showed the same degree of PRBC sequestration within different parts of the brain from individual monkeys. Cerebral edema could not be conclusively demonstrated and no cerebral hemorrhages were seen in these brains. Electron microscopy demonstrated multiple electron dense knobs protruding from the membrane of PRBC. These knobs cytoadhered to endothelial cells of cerebral microvessels.

Immunohistochemistry was performed using antisera against IgG and C3, and antisera or monoclonal antibodies (Mabs) against CD36, TSP, ICAM-1. IgG and C3 were found in the cerebral microvessels of all monkeys infected with *P. coatneyi*. On the other hand, CD36,

TABLE III

Immunofluorescent localization of various antigens in cerebral microvessels (antibody-specific reactivity)

	IgG	C3	CD36	TSP	ICAM-1
AF - B464	+	+	+	+	+
AF - G425	+	+	+	+	+
AF - B298	+	+	–	–	–
CDC - 3	+	+	+	+	+
CDC - 838	+	+	+	+	+
3927 (control)	–	–	–	–	–
233 (control)	–	–	–	–	–

+: positive reaction.
–: negative reaction.

TSP and ICAM-1 were observed in AF-B464, AF-G425, CDC-8 and CDC-838, but not in AF-B298 or the control monkeys (Table III).

DISCUSSION

In order for an animal system to serve as a model for human cerebral malaria it is essential that sequestration of PRBC occur in cerebral microvessels. Although rodents infected with *P. yoelii* or *P. berghei* have been used to study cerebral malaria, these systems appear to have little relevance to human cerebral disease because of differences in the pathogenesis of the infection (Grau et al., 1990). In *Plasmodium* - infected rodents, macrophages and lymphocytes accumulate in the microvessels, and PRBC sequestration and cytoadherence of PRBC via knobs in the membrane of infected erythrocytes does not occur.

Plasmodium coatneyi, a malaria parasite of macaque monkeys, produces knobs on the membrane of PRBC and our electron microscopic study has demonstrated that these knobs mediate cytoadherence of PRBC to host endothelial cells. Although monkey AF-B298 showed sequestration of PRBC in only 4% of cerebral microvessels, all the other animals demonstrated substantial degrees of sequestration. When the brains of human cerebral malaria cases were studied, 96% of cerebral microvessels showed sequestration of PRBC and the brains of non-cerebral falciparum human malaria cases showed sequestration of PRBC in 13% of cerebral microvessels (Riganti et al., 1990). The degree of sequestration in rhesus monkeys was not as prominent as in cerebral malaria patients. It is possible that occlusion of cerebral microvessels in these monkeys was below the threshold needed for appearance of clinical signs such as seizure and coma. As Table I shows, AF-B464 and AF-G425 have PRBC sequestration in 40% and 35% of cerebral microvessels respectively. The peripheral blood of these monkeys at the time of death contained almost no schizonts. This may indicate that most schizont-infected RBC's were sequestered on the various organs including the brain. On the other hand, AF-B298 had PRBC sequestration in only 4% of cerebral microvessels although the peripheral blood showed no schizont infected RBC's. Immunofluorescent microscopy failed to show CD36, TSP and ICAM-1 in cerebral microvessels of this monkey. This may indicate that this particular monkey dit not express these cytoadherence proteins in brain microvessels even though they may have been expressed in other tissues. CDC-3 and CDC-838 showed 58% and 52% of the total parasites as schizont-infected erythrocytes in the peripheral blood respectively and PRBC sequestration in 29% and 28% cerebral microvessels. The high number of schizont-infected erythrocytes in the peripheral blood may relate to the fact that these monkeys were splenectomized prior to *P. coatneyi* infection. A similar observation was described in splenectomized chimpanzees infected with *P. falciparum* (Hickman, 1969). Splenectomy may result in the loss or decrease in the expression of the adherence ligand on PRBC.

Cytoadherence molecules of host cells such as CD36, TSP and ICAM-1 have been intensively studied by various investigators. Barnwell et al. (1985) showed that CD36 is a receptor for a ligand on the surface of *P. falciparum* – infected erythrocytes and antibody OKM 5 against CD36 blocked the cytoa-

dherence of PRBC. Roberts et al. (1985) have shown that TSP, independent of other ligands, could play a role in the cytoadherence of knobs. Berendt et al. (1989) reported that an endothelial cell-binding line of *P. falciparum* binds to COS cells transfected with ICAM-1. An ICAM-1 Mab blocked adhesion of PRBC to the transfected COS cell line.

Our study demonstrated a correlation between sequestration of PRBC and the presence of CD36, TSP and ICAM-1 in the cerebral microvessels of *P. coatneyi* - infected rhesus monkeys. Control uninfected animals and one monkey with PRBC sequestration in only 4% of cerebral microvessels did not show the presence of these molecules in brain. This finding strongly suggests that these molecules are responsible for PRBC sequestration (Aikawa et al., 1992). It is not clear whether some monkeys naturally express these molecules and are more susceptible to cerebral complications or whether there is an as yet unknown trigger that induces expression of these molecules during malaria infection.

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