Human Cerebral Malaria

A Quantitative Ultrastructural Analysis of Parasitized Erythrocyte Sequestration

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For investigation of the pathogenesis of cerebral malaria, immediate postmortem samples from brain and other tissues of patients dying with *Plasmodium falciparum* malaria, with (CM) or without (NCM) cerebral malaria, were processed for electron microscopy. Counts of parasitized erythrocytes (PRBCs) in cerebral and other vessels showed that the proportion of PRBCs was higher in CM than in NCM, and also that the proportion of PRBCs was higher in the brain than in other organs examined in both CM and NCM. Cerebral vessels from CM patients were more tightly packed with RBCs than those from NCM patients, but there was no significant difference in the amount or degree of endothelial damage or numbers of vessels with endothelial pseudopodia. Fibrillar (fibrin) deposits were present in a small proportion of vessels, but no thrombosis was present. There was neither acute nor chronic inflammation, and leukocytes were absent within or outside cerebral vessels. There was no immune complex deposition in cerebral vessels. Parasites in cerebral vessels were mainly trophozoites or schizonts. Occasional RBC remnants following parasite release were seen. Some parasites were degenerate, resembling crisis forms. PRBCs adhered to endothelium via surface knobs. It is concluded that there is no evidence for an inflammatory or immune pathogenesis for human cerebral malaria and that the clinical effects probably relate to anoxia and the metabolic activities of the parasites (Am J Pathol 1985, 119:385–401)

CEREBRAL MALARIA is a diffuse symmetric encephalopathy occurring in a proportion of patients infected with *Plasmodium falciparum*. It is the most important severe manifestation of *P falciparum* infection and carries a mortality of between 20% and 50%.[1,2] The clinical features of cerebral malaria are well documented,[3–7] but some aspects of its pathogenesis remain obscure. Several mechanisms have been proposed. These include mechanical obstruction of cerebral vessels resulting from the decreased deformability of parasitized erythrocytes (PRBCs)[8] or from the adhesion of PRBCs to vascular endothelium[9,10]; the release of toxic factors from PRBCs, leading to increased vascular permeability, the breakdown of the blood–brain barrier, and cerebral edema[11,12]; and the induction of an inflammatory response in and around cerebral vessels, leading to increased vascular permeability and cerebral edema.[13]

The pathology of cerebral malaria in humans has been reviewed at gross and light-microscopic levels,[14] and ultrastructural studies of the pathologic features of human *P falciparum* infection in the liver and kidneys have been reported.[15,16] No animal model has successfully mimicked the characteristic clinical and pathologic features of human cerebral malaria, but detailed ultrastructural studies have been made of the pathologic features of *P falciparum* in the owl monkey, *Aotus*.[17] Several studies have described cerebral disease in rodents following infection with various species of *Plasmodium*.[13,18] In the majority of these reports, the disease is related to inflammatory intravascular and

Supported by the Wellcome Trust of Great Britain as part of the Wellcome–Mahidol University, Oxford Tropical Medicine Research Programme.

Accepted for publication January 16, 1985.

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perivascular changes. The relationship of these models to human cerebral malaria is not clear.

Any explanation of the etiology of human cerebral malaria must take into account the morphological as well as the clinical features of the disease. Previous studies have examined routine autopsy specimens, where the possibility of significant postmortem degeneration could not be excluded. The present study was undertaken in an attempt to detect differences between the ultrastructure of freshly fixed specimens of brain and some other organs in patients dying from cerebral malaria and control subjects dying with noncerebral malaria. Specimens were examined for specific features with a semiquantitative procedure, and the results were correlated with the clinical diagnosis.

The sequestration of red cells containing mature forms of the parasite in capillaries and postcapillary venules demonstrated in this study appears to be a consistent feature of severe *P falciparum* malaria. This results in large discrepancies between peripheral blood parasite counts and the parasite count observed in histologic specimens—these discrepancies are most evident in cerebral malaria. Blood smears taken from capillary finger-prick or venous samples may therefore considerably underestimate the total parasite load in the infected patient. The possibility that sequestration might occur in the dermal capillaries, and that parasite counts obtained from skin smear or skin biopsies would therefore more accurately represent the total parasite load than conventional blood smears was investigated in patients with cerebral and uncomplicated *P falciparum* malaria.

Materials and Methods

Clinical

Seven patients with cerebral malaria who died in the intensive care unit of PraPokklao Hospital, Chantaburi, Eastern Thailand, were studied. Investigation and management of these patients has already been described.[2] All were treated with parenteral quinine. Cerebral malaria was defined as unrousable coma in a patient with asexual forms of *P falciparum* detectable in the peripheral blood smear. Other causes of coma were excluded. Clinical, biochemical, and hematologic details of these cases are given in Table 1. Control specimens were taken from 6 patients who died during the course of a *P falciparum* infection, but without cerebral malaria.

The study of skin sequestration of PRBCs included nine patients with cerebral malaria and seven with uncomplicated *P falciparum* malaria whose venous blood parasitemia exceeded 1%. Informed consent to skin biopsy was obtained from the patients or their accompanying relatives. These patients were studied as soon after admission to the hospital as possible.

Collection and Fixation of Specimens

Samples of tissues were taken from patients within 90 minutes of death. Brain tissue was obtained from every case and kidney, liver, lung, and heart tissue was obtained from some patients with a Franklin-modified vim Silverman biopsy needle. Pieces were discharged, cut into 1-mm cubes with a scalpel, and fixed. Areas of gray matter in brain specimens were selected for fixation, and smears were also made on microscope slides and stained with Giemsa or Wright's stain. Blood samples were discharged into stirred fixative. Fixation was with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, at 4 C for 2 hours. Samples were then washed three times, coded, and flown to Oxford in a buffer solution. All subsequent processing was done without knowledge of the clinical diagnosis. Specimens were postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate, pH 7.4, dehydrated in graded ehtanols, and embedded in Araldite. One-micron sections were stained for light microscopy.[19] Thin sections cut with a diamond knife and stained with lead citrate[20] were examined on a JEOL 100XC electron microscope. Some specimens were stained prior to postfixation to reveal endogenous peroxidatic activity[21] with the use of diaminobenzidine (1 mg/ml) in Tris-HCl buffer, pH 7, containing 0.005% H_2O_2.

Capillary blood samples were obtained by pricking the finger pulp with a 25-gauge needle. Venous samples were obtained from the antecubital veins. Standard thin films were prepared. Slit skin smears were obtained from the lobe of an ear, as described for the diagnosis of leprosy. Skin biopsies were taken with a standard 4-mm circular punch. Care was taken not to squeeze the skin beforehand. Local anesthetic was infiltrated subcutaneously. The base of the biopsy sample was then smeared repeatedly on a glass slide. Duplicate slides were prepared and stained with Giemsa. The number of parasites in 2000 red cells was counted on each slide. The results were compared by paired t tests.

Analysis of Sections

Thick (1-μ) sections of brain and other tissues were examined by light microscopy. Blood vessels were identified, intravascular RBCs were counted, and the proportion containing recognizable asexual parasites was scored.

Thin sections were examined systematically with the squares on the support grids as reference areas. At least 20 blood vessels (or all vessels on small or sparsely vas-

Table 1—Clinical Details of Patients With *P falciparum* Malaria

Age, sex (months pregnant)	Day of fever	Retinal hemorrhage	CSF opening pressure (cm)	Hematocrit (%)	WBC $\times 10^3$/l	Platelets $\times 10^3$/l	Parasitemia/l	Fibrinogen (mg/dl)	FDP (μg/ml)	Blood urea nitrogen (mg/dl)	Serum creatinine (mg/dl)	Total bilirubin (mg/dl)	Serum aspartate aminotransferase (Reitman–Frankel units/ml)	Hypoglycemia	Other complications
Fatal cerebral malaria: brain and other biopsies															
1 16F	?	+	–	–	–	–	0 (brain + smear)	–	–	–	–	–	–	+	Hypotension, pulmonary edema
2 31F	4	+	160	17	17.7	25	73,876	217	24	124	8.0	6.2	516	–	Hypotension, pulmonary edema
3 36M	3	–	30	28	22.0	54	85,625	585	12	113	6.0	17.6	390	+	Hypotension, acute renal failure (peritoneal dialysis)
4 16M	4	–	–	27.5	19.0	57	76,720	475	24	162	2.0	2.1	27	–	Hypotension, supraventricular tachycardia
5 19F	6	–	–	28	8.1	123	639,937	330	6	56.6	3.7	–	–	+	–
6 42M	3	–	–	37	11.6	57	638	368	6	14.2	1.2	1.2	45	–	Aspiration pneumonia
7 23F (7)	6	–	–	12.5	11.3	24	79,605	126	24	57	3.3	0.33	127	–	Pulmonary edema
Fatal noncerebral malaria: brain and other biopsies															
8 45M	?	–	–	–	–	–	+	–	–	–	–	–	–	–	Cardiac arrest 3 hours after admission
9 58F	5	–	–	20	19.1	–	"Many"	–	–	74	4.7	4.6	41	+	Hematemesis, hypotension
10 38F (9)	6	–	–	22	14.2	36	9,442	–	–	140	5.8	6.1	73	+	Hypotension, septicemia, pneumonia, pulmonary edema
11 45F	2	–	–	22	14.0	202	526,414	217	–	73	5.8	2.0	93	+	Hypotension, acidosis
12 64M	7	–	–	10.5	12.1	–	"Few"	–	–	–	–	–	–	–	–
13 16F	1	–	–	27	5.0	–	"Moderate"	–	–	34	1.9	2.8	41	–	Massive aspiration
Cerebral malaria (*fatal case): skin smears and biopsies															
14 28F (3)	5	+	130	18	11.1	41	127,800	280	7.5	30	1.3	8.5	164	–	–
15 23F	5	+	–	26	15.9	–	76,631	243	7.5	80	4.5	9.2	350	+	Hematemesis
16 21F (6)	4	–	120	17	8.4	140	24,108	205	7.5	15	2	1.8	45	–	–
17 37M	3	–	148	28	11.5	77	129,280	243	7.5	50	2.3	2.2	49	–	–
18 24M*	4	+	–	19.2	19.0	85	34,625	160	<3	171	9.0	20.8	290	+	Hematemesis, hypotension, aspiration pneumonia
19 20M	4	–	142	29	10.8	55	3,700	230	<3	22	2.5	0.8	128	–	–
20 25M	5	+	214	30	10.9	53	14,300	260	60	138	14	14.6	164	–	–
21 24M	10	+	95	19	13.1	112	74,670	72	15	69	1.7	0.7	53	–	–
22 19M	2	–	92	31	9.3	129	1,320	192	<3	16.2	1.3	0.9	27	–	–

Table 2—Comparison of Cerebral Vessels From Patients With and Without Cerebral Malaria*

Patient	% RBCs parasitized	Degree of packing (0–3)	% Vessels with 3+ packing	Extravascular RBCs (no. of occasions, not individual cells)	% Vessels with endothelial damage	% Vessels containing PRBCs	% Vessels with dense deposits
Group 1: cerebral malaria							
1	34.1	1.6	25.0	0	93.7	25.0	6.3
2	25.2	1.23	25.9	4	26.9	33.3	3.9
3	53.0	2.58	65.0	2	30.0	70.0	0
4	55.0	2.82	90.9	1	100.0	100.0	9.1
5	48.3	2.67	77.8	3	55.5	80.0	44.4
6	43.8	2.69	76.9	0	29.4	76.9	0
7	43.8	2.11	50.0	0	70.0	55.5	7.1
Mean ± SD	43.3 ± 10.6	2.24 ± 0.62	58.8 ± 26.0		57.9 ± 40.0	63.0 ± 26.7	10.1 ± 15.5
Group 2: noncerebral malaria							
8	0	0.47	0	0	70.0	5.0	0
9	30.0	1.25	25.0	0	12.5	25.0	0
10	7.0	0.13	0	0	12.5	0	12.5
11	52.5	1.25	12.5	1	58.8	52.9	0
12	4.7	0.67	0	1	20.0	5	4.8
13	5.5	0.50	8.3	0	0	0	0
Mean ± SD	16.6 ± 20.5	0.71 ± 0.45	7.63 ± 10.0		29.0 ± 28.4	14.65 ± 20.9	2.88 ± 5.09
T	2.15	2.72	2.95	1.25	1.79	2.63	1.20
P	<0.05	<0.01	<0.01	NS	NS	<0.01	NS

* Specimens from individual patients were coded before examination and scored without knowledge of the clinical diagnosis. The significance of differences between the groups was tested with the Mann–Whitney test.

cularized specimens) were identified and scored for a variety of characteristics. These included

1) Type of vessel (arteriole, capillary, or venule)
2) Presence of PRBCs (+ or −)
3) Tightness of packing of RBCs (scored as 0–3+)
   0 = no RBCs
   1+ = loose, free-floating RBCs
   2+ = vessel filled with RBCs but little distortion of cell outlines
   3+ = vessel filled with RBCs with obvious distortion of cell outlines
4) State of RBCs (density of cytoplasm, presence of surface excrescences [knobs], apparent adhesion to endothelium)
5) State of endothelium (intact or damaged, presence of gaps between endothelial cells, presence of pseudopodia, presence of deposits on basement membrane)
6) State of parasites ("healthy" or "damaged," state of maturation)
7) Presence of leukocytes and platelets
8) Presence of extravascular RBCs
9) Presence of intravascular deposits

Controls

Apart from the samples obtained from *P falciparum*-infected patients dying without overt cerebral malaria, specimens obtained at open brain surgery and fixed immediately in glutaraldehyde were also examined (kindly supplied by Dr. M. Esiri, Radcliffe Infirmary, Oxford).

Statistical Analysis

Data from the different groups were compared with the Student *t* test or the Mann–Whitney test.

Results

Clinical Details

Clinical details of the 30 patients studied are given in Table 1.

Preservation of Specimens

In general, the tissues showed very good preservation of ultrastructural detail in comparison with specimens obtained during open brain surgery. Artifacts were present in some cases—eg, neuronal degeneration, gaps between blood vessels and surrounding brain tissue, and damage to renal tubular epithelium—but blood vessels and their contents were generally well preserved. In particular, membranes and mitochondria showed few signs of artifactual damage.

Comparison of Brain Specimens From Patients With (CM) or Without (NCM) Cerebral Malaria

Sections of brain from *P falciparum*-infected patients were examined "blind," as described above, and scored for abnormalities. Those abnormalities amenable to semiquantitative analysis are presented in Table 2 and

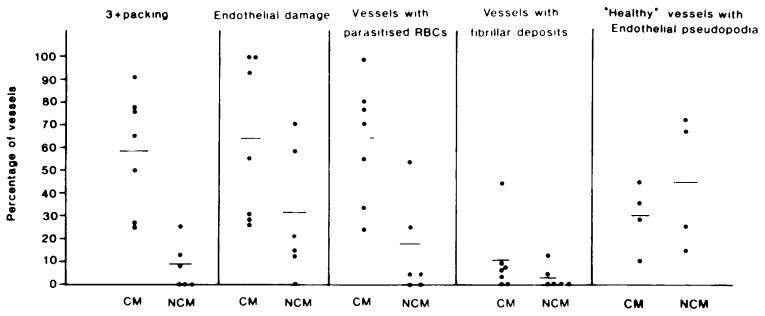

Figure 1—Comparison of blood vessels from patients with and without cerebral malaria. Electron micrographs of vessels from CM and NCM patients were scored "blind" for several parameters. The *points* represent the average score for each parameter in an individual patient. The *bars* represent means for all patients.

Figure 1. Several major differences between the two groups are apparent:

1) More than three times as many vessels in the CM group contained PRBCs ($P < 0.05$). The great majority of parasitized vessels were capillaries and venules. Only occasional arterioles were seen, and these were almost always empty of cells.

2) The average tightness of RBC packing in vessels (on a 0–3+ score) was significantly higher in the CM group (2.24) than in the NCM group (0.71) ($P < 0.005$).

3) The percentage of tightly packed vessels (3+) was significantly higher in the CM group (58.7%) than in the NCM group (7.6%) ($P < 0.001$).

4) Endothelial damage was observed in many specimens. In CM, the proportion of vessels showing damage ranged from 27% to 100% and in NCM from 0% to 58% but the difference is not statistically significant. Few gaps were seen between endothelial cells in undamaged vessels, and there was no difference in their frequency between the groups.

5) Ten percent of the vessels in the CM group contained dense fibrillar deposits (range, 0–44%), whereas

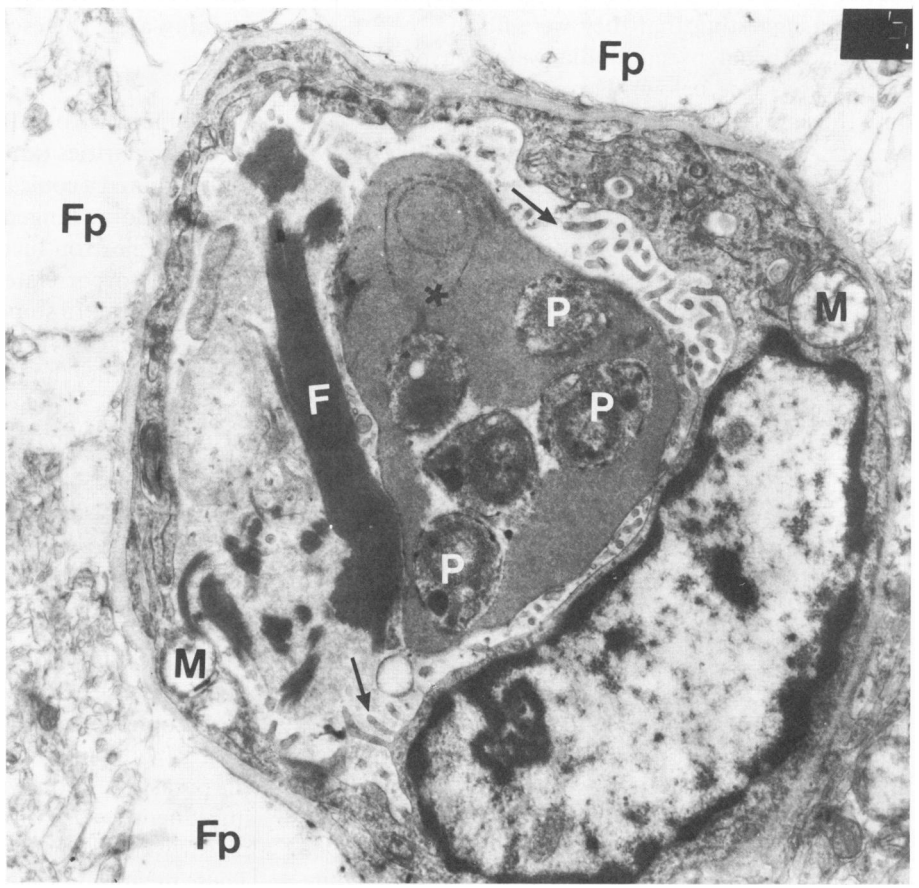

Figure 2—Cerebral malaria. A parasitized RBC within a cerebral venule. The parasites are undergoing schizogony (*P*), and membranes appear to be attached to the parasitophorous vacuole at one point (*). Dense fibrillar material is present within the lumen of the vessel (*F*). The endothelium is relatively undamaged, but mitochondria are not well preserved (*M*). Numerous fingerlike endothelial pseudopodia are present (→). Astrocyte footpads are swollen (*FP*). (×10,000)

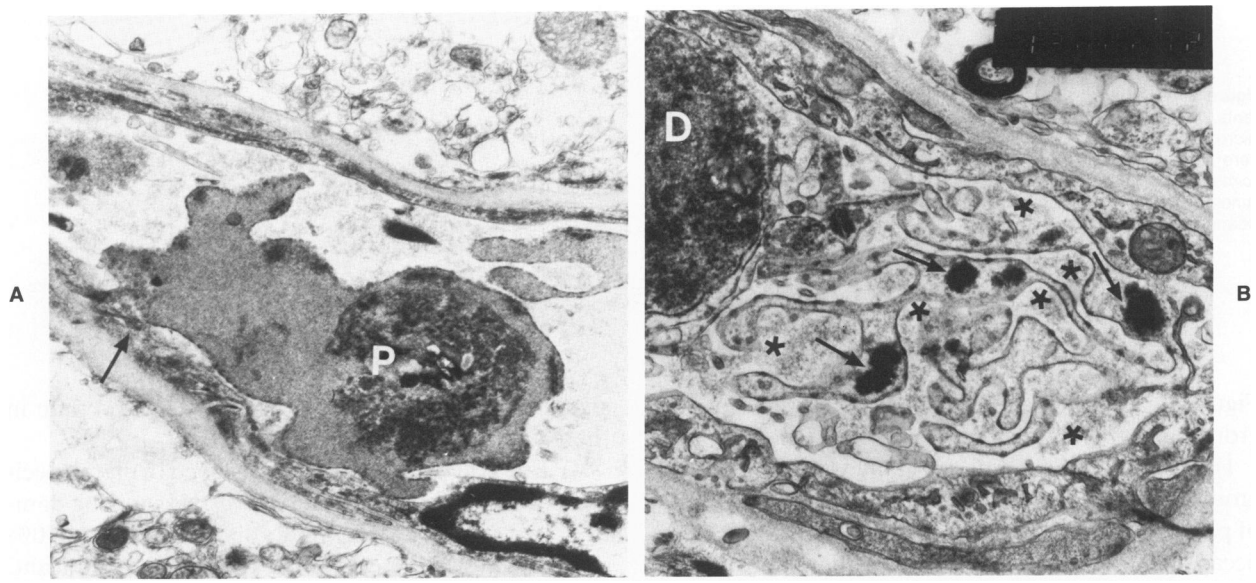

Figure 3A—Cerebral malaria. An irregularly shaped parasitized RBC in a cerebral venule. The RBC shows numerous surface knobs. The parasite appears degenerate (*P*). The vascular endothelium is damaged close to the apparent point of attachment (→). (×9000) **B**—Cerebral malaria. A presumed "ghost" of a parasitized RBC within a cerebral venule. The "ghost" is recognizable as such because of the numerous surface knobs. Some dense deposits are present within the cell (→). Extracellular spaces are shown (*). The nature of the large dense body (*D*) is unknown. (×16,900)

only two vessels in all the NCM specimens contained similar deposits. On some occasions the deposits were striated, suggesting that they were fibrin.

6) Endothelial pseudopodia were conspicuous in some vessels (see below). For comparative purposes, only those vessels with undamaged endothelium were scored. A higher proportion of vessels in the NCM group possessed pseudopodia, but the difference was not statistically significant ($P > 0.5$). Two patients in the NCM group (Patients 9 and 11) differed conspicuously from the rest of the group in terms of the percentage of parasitism of RBCs, the tightness of packing of cerebral vessels, and the proportion of vessels containing PRBCs. Although in coma, their condition was not diagnosed as CM because of other clinical features.

Structure of PRBCs

PRBCs displayed several distinct abnormalities.

Shape

Some PRBCs possessed relatively regular outlines (Figure 2), but the majority were very irregular. The degree of irregularity appeared to correlate with the time after parasitization, in that the most bizarre outlines and largest parasites were present in cells with the least remaining hemoglobin, as judged by cytoplasmic electron density and peroxidatic activity (Figure 3A). Occasionally cells were seen with extremely distorted outlines (Figure 3B). They were recognizable as RBCs only because of the presence of many knobs on their surface. They are thought to represent RBC "ghosts" remaining after the escape of parasites.

Plasma Membrane

The majority of PRBC possessed conspicuous surface irregularities (knobs). When fully developed, the knobs showed a conical outline with a circular base (Figure 4A). Some tangential profiles of knobs showed a relatively electron-lucent core. The numbers of knobs appeared to correlate with the length of time after parasitization. In some cells the knobs were rather regularly arranged, with a similar gap between knobs. On occasion, rather than discrete knobs, there appeared to be larger areas of dense material underlying the plasma membrane, perhaps due to the coalescence of individual knobs (Figure 4B). In many instances it was difficult to follow the bilayered plasma membrane because of tangential sectioning, but where it was distinct, it was clearly overlying the knobs (Figure 4C).

Cytoplasm

The cytoplasm of parasitized cells was usually less electron-dense than that of normal RBCs. In many cases the cytoplasm had a granular appearance (see Figure 3A). Few organelles were seen in PRBCs apart from the parasitophorous vacuole. Maurer's clefts were seen infrequently. Occasionally, volumes of PRBC cytoplasm were seen to be enclosed within membranes. These membranes showed the typical trilaminar structure but appeared to be discontinous. It is not clear

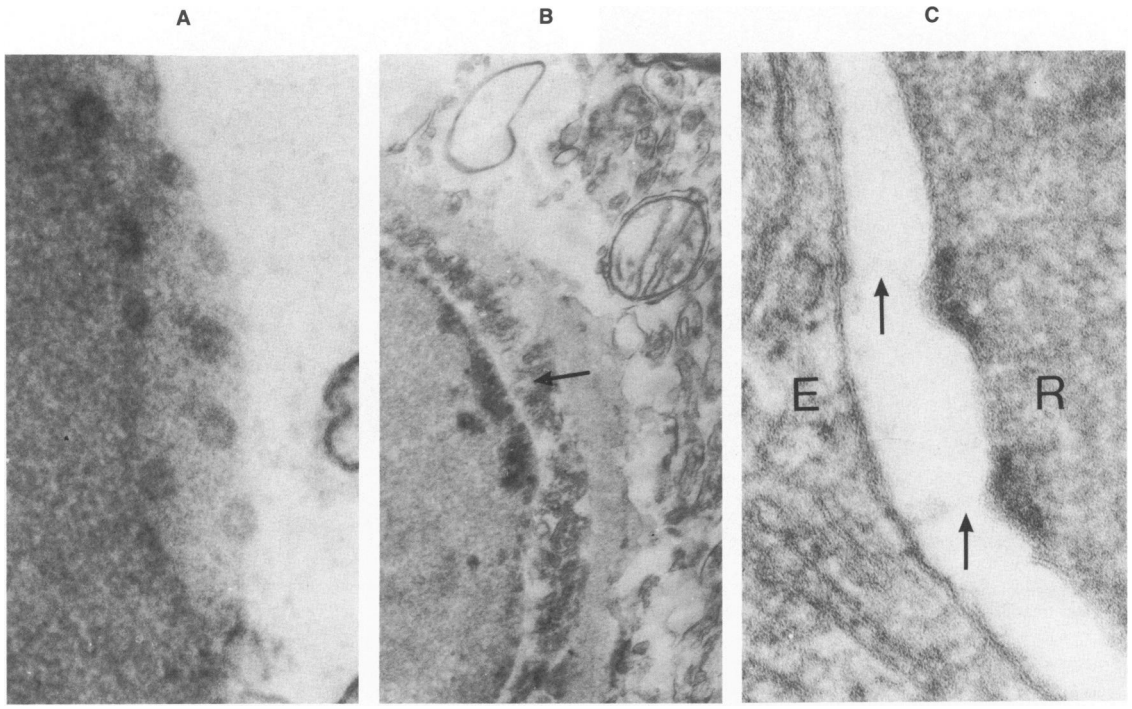

Figure 4A—Cerebral malaria. Tangential section through the edge of a parasitized RBC. Surface knobs are separated by regular gaps. They appear to have a less electron-dense central core. (×80,000) **B**—Cerebral malaria. A cerebral venule tightly packed with parasitized RBC. The endothelium (→) is almost totally destroyed. Dense material is aggregated under the RBC plasma membrane. (×16,000) **C**—Cerebral malaria. Cerebral venule. Strands of electron-dense material (↑) connect the endothelial cell (*E*) with the RBC (*R*). The plasma membrane of the RBC clearly overlies the knobs. (×132,000)

whether this represents natural fenestration or a postmortem artifact. In some cases the membranes were single (Figure 5A), but in many cases they consisted of two distinct trilaminar layers (Figure 5B). These membranes usually showed no connection with the parasitophorous vacuole or the cell surface, but occasionally they were clearly attached to the parasitophorous vacuole (Figures 5A and 6A). On one occasion multiple membranes were seen to be attached to the vacuole (Figure 6A). At the site of attachment a complex arrange-

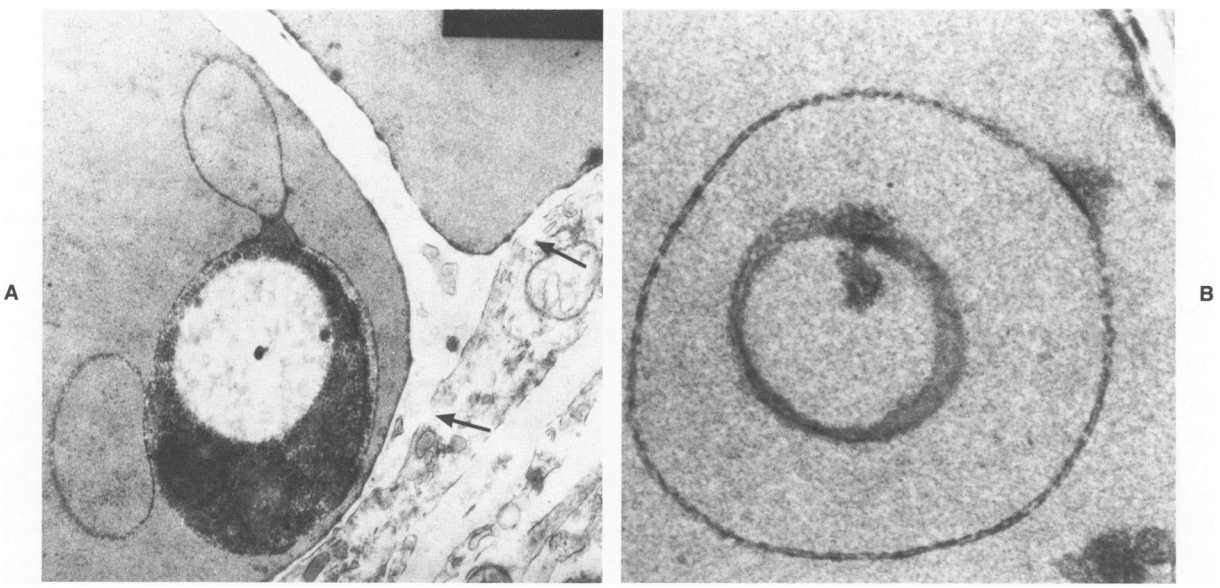

Figure 5A—Trophozoite in an RBC. Volumes of cytoplasm are enclosed by apparently fenestrated membranes. One membrane is clearly attached to the parasite. The endothelium shows postmortem damage (→). (×20,800) **B**—Membranes within PRBCs. The outer set of membranes is clearly double. The inner membranes are sectioned tangentially. Electron-dense material is associated with both membranes. (×46,200)

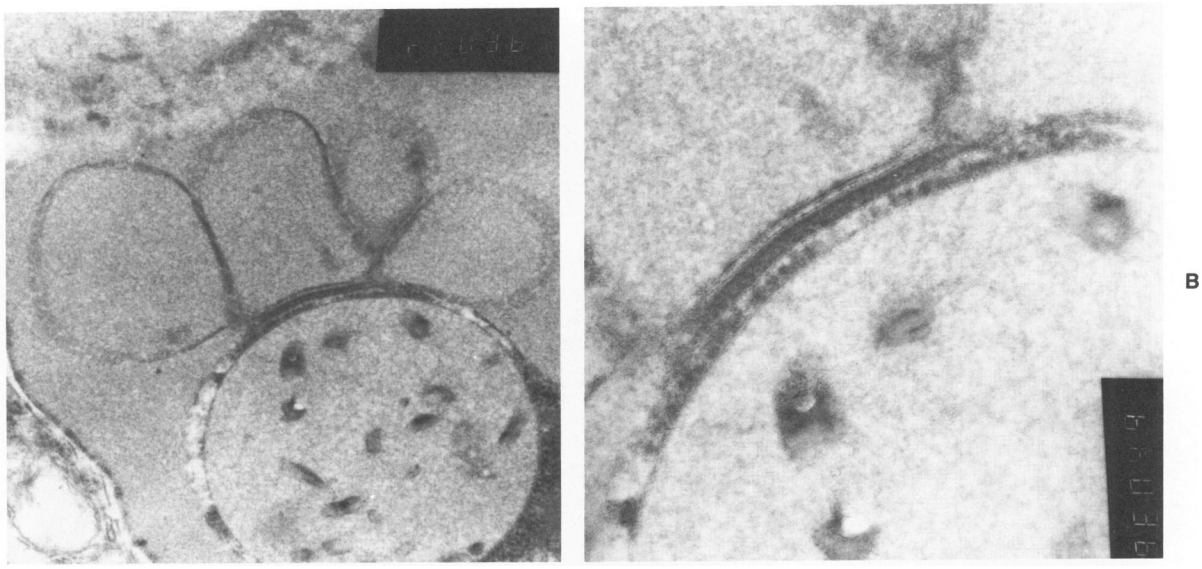

Figure 6A—Four sets of membranes are associated with a single trophozoite. (×33,800) **B**—Complex arrangement of membranes adjacent to parasite. Five distinct trilaminar membranes can be distinguished (detail of 6A). (×99,000)

ment of membranes and electron-dense and lucent layers was apparent (Figure 6B).

No direct connections with the plasma membrane were observed, but on several occasions a band of electron-dense material was seen to be connected to the outer surface of the membranes and to approach the plasma membrane of the PRBCs (Figures 5B and 7A–C).

Parasites

The structure of *P falciparum* in infected RBCs has been described previously,[22] and no major differences were noted in this study. All stages of parasite development from early trophozoite to late schizont could be seen, but the majority were late trophozoites or schizonts, and in many instances the parasites showed

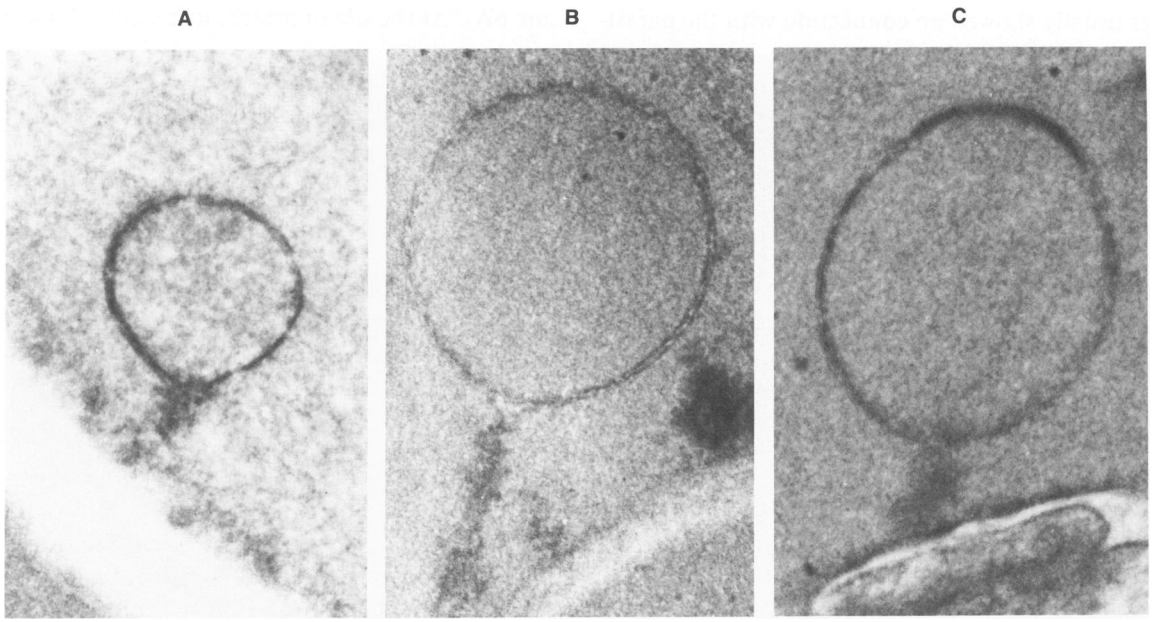

Figure 7A–C—Examples of electron-dense material between cytoplasmic membranes and plasma membrane in PRBCs. (**A**, ×52,800; **B**, ×49,400; **C**, ×75,000)

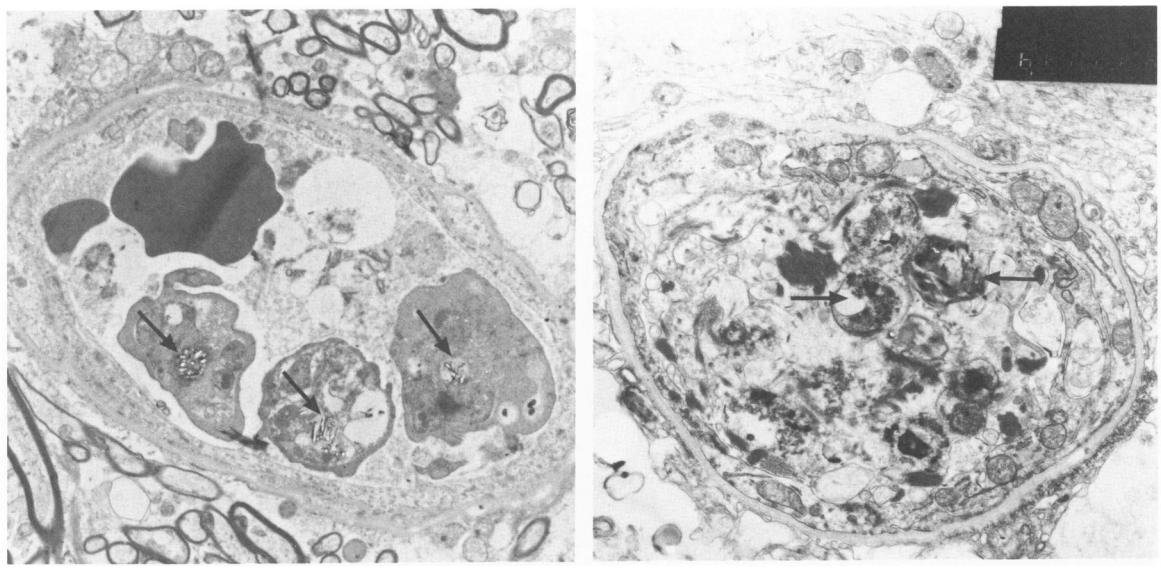

Figure 8A—Three PRBCs showing schizonts in a cerebral blood vessel. Pigment granules are conspicuous (→). (×5000) **B**—Cerebral malaria. A cerebral venule is filled with a mass of degenerating material in which parasitic remains including malarial pigment are clearly visible (→). The endothelium of the venule appears to be intact. (×8600)

morphologic signs of degeneration (Figure 3A). In many instances pigment was clearly present within the parasite (Figure 8A). On occasion, vessels appeared to be blocked by a mass of necrotic material in which parasites and RBCs could both be identified (Figure 8B). In these vessels, the parasites and RBCs showed more advanced degeneration than the blood vessels, indicating that the degeneration was not a postmortem artifact. These degenerate parasites are somewhat similar morphologically to "crisis forms."

Interaction of RBCs With Vascular Endothelium

It has been suggested previously that PRBCs may adhere to endothelial cells by their "knobs."[9,10] We sought evidence to test this hypothesis. The observations were as follows:

1) PRBCs were seen in intimate contact with endothelial cells at sites with and without knobs.

2) In the majority of points of contact a gap was clearly visible between plasma membranes of the PRBCs and endothelial cells. Where no gap was visible, it seemed likely that this was the result of tangential sectioning. No clear evidence for membrane fusion was obtained.

3) On several occasions, the contours of PRBC followed closely those of an endothelial cell but with a relatively large gap, probably due to shrinkage during fixation. In such cases, strands of electron-dense material could sometimes be seen running between the two cells, and the point of origin of such strands on the PRBC was very frequently a knob or area of increased density (Figure 4C).

In an attempt to show that such strands arose preferentially from knobs, the points of origin of electron-dense strands between PRBCs and endothelial cells were counted. On electron micrographs of 10 randomly chosen PRBCs displaying knobs, measurements were made of the length of plasma membrane in the profile, the number of knobs, and the average length of the knobs. On average, knobs occupied 21.6% (±5.5%) of the cell perimeter, whereas 64.1% of electron-dense strands ($n = 64$) arose from knobs, thus showing a 3-fold increase in the observed, compared with expected, frequencies.

4) In the majority of cases, the endothelial cells showed no changes at the point of contact, but occasionally an area of increased density was observed. Sometimes the endothelium appeared to be damaged opposite a PRBC knob, and it is thought that this may represent damage occurring during tissue processing because of the adhesion of PRBCs to endothelium.

Cells

Occasional neutrophils were seen, but other leukocytes were not observed in significant numbers either in or outside blood vessels. A striking negative observation was the total absence of blood platelets, either circulating or in thrombi.

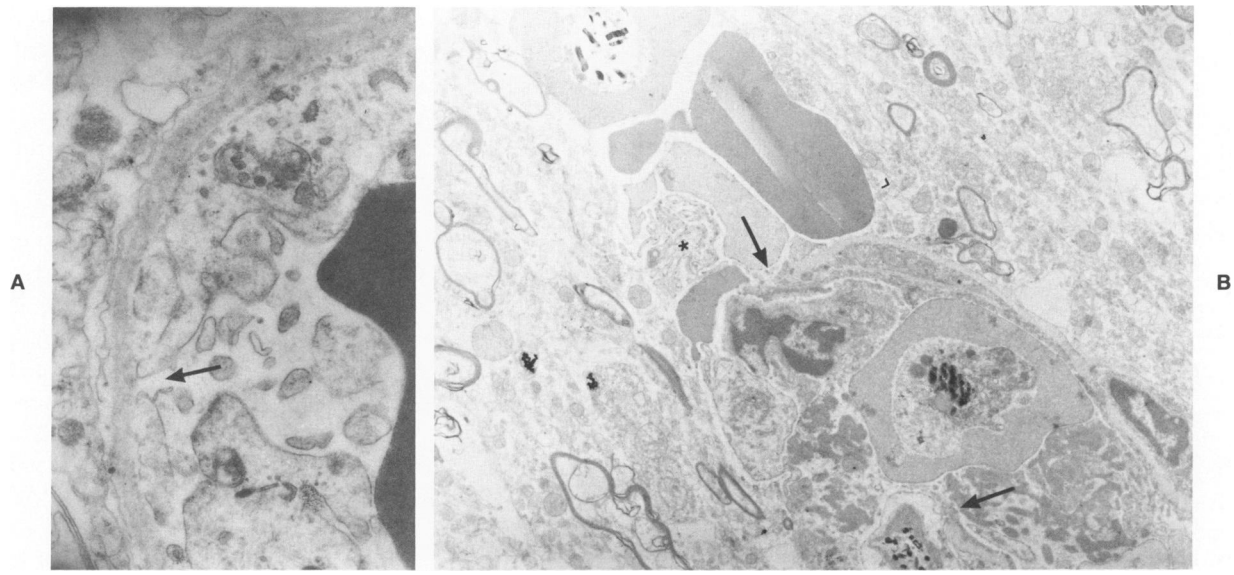

Figure 9A—Cerebral malaria. The endothelium of a cerebral venule is damaged, and a gap (→) extends to the basement membrane (*B*). (× 18,200) **B**—Cerebral malaria. A ruptured cerebral venule has allowed the escape of PRBCs. The ends of the venule wall are marked by *arrows*. A PRBC ghost is present (*). (× 4300)

Endothelial Cells

In many specimens, endothelial cell morphology was well preserved, but in some vessels the cells showed advanced degenerative changes with loss of the luminal membrane and disorganization of the cytoplasm (Figure 4B). In some specimens, all vessels showed such changes; but in others, vessels within 100 μ of a damaged vessel were unaffected, indicating that the degeneration was unlikely to be postmortem. Occasionally, gaps in otherwise healthy endothelium could be seen, and in some cases the basement membrane was exposed (Figure 9A).

On only one occasion was a total defect in a venule wall observed (Figure 9B). In this case, PRBCs had escaped in small numbers into the surrounding tissue. No platelets were seen in the damaged area.

A striking feature of endothelial cells in many vessels from patients with and without cerebral marlaria was the presence of numerous projections extending

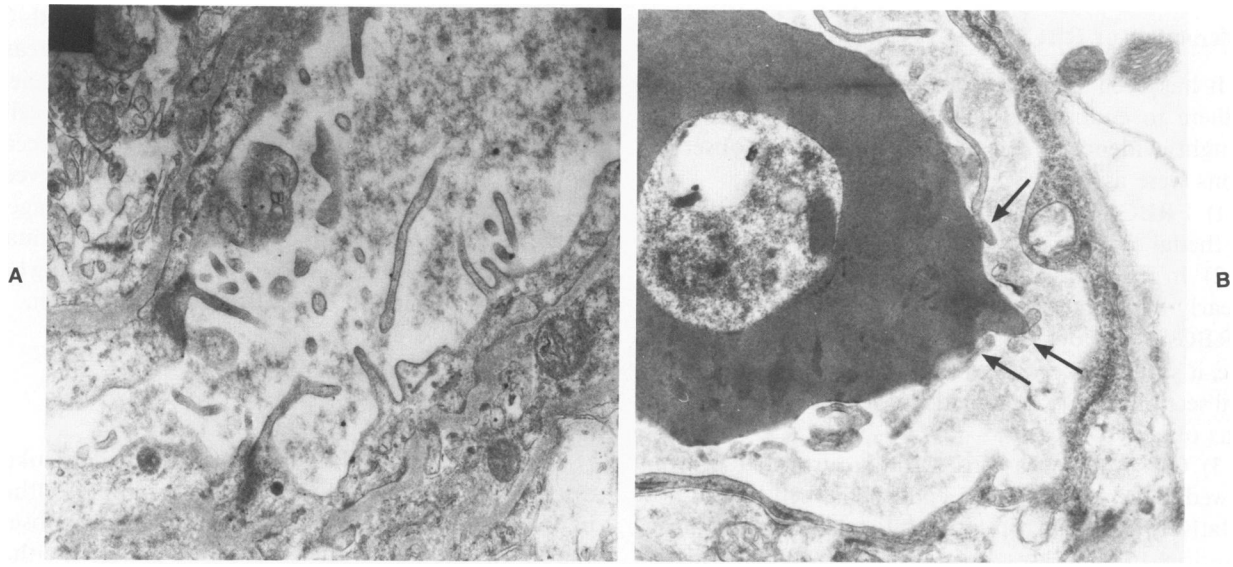

Figure 10A—Noncerebral malaria. Numerous fingerlike pseudopodia in a cerebral venule. (× 13,000) **B**—Cerebral malaria. Endothelial pseudopodia are apparently attached to a PRBC in a cerebral venule (→). (× 10,000)

Table 3—Frequency of PRBCs in Different Organs*

Organ	Cerebral malaria	Noncerebral malaria
Brain	45.1 (2185) (7)	19.4 (643) (6)
Heart	19.8 (333) (3)	23.5 (34) (1)
Lung	4.2 (568) (4)	35.7 (14) (1)
Liver	17.0 (200) (1)	0.67 (150) (1)
Kidney	4.70 (830) (5)	5.03 (397) (3)

* Percentage of RBCs parasitized. Total number of RBCs counted is in first set of parentheses, followed by the number of patients.

One-micron sections were examined with light microscopy. Twenty vessels, or all vessels if the specimen was small, were examined. The total number of RBCs and the proportion containing parasites were counted.

into the lumen of the vessel (Figure 10A). In some cases, such projections were seen in vessels apparently blocked by RBCs, and on many occasions the projections were in intimate contact with the parasitized cells (Figure 10B). Infrequently, endothelial cell projections were seen in vessels from control specimens, but these were never as frequent nor as long as in malarial specimens.

Basement Membrane

The basement membrane was not noticeably thicker in blood vessels containing PRBCs than in control vessels, and there was no sign of deposits resembling immune complexes.

Frequency of Parasitized RBCs in Brain and Other Organs

Araldite-embedded sections 1μ thick were examined under oil immersion, and all blood vessels within the section were identified. Profiles of all intravascular RBCs and the proportion containing recognizable parasites were counted. Two hundred RBCs per section or the total number in sections containing less than 200 was counted. The results are shown in Table 3. Figure 11 shows the relationship between the proportions of PRBCs in brain and other organs where samples were available from the same patient. The lines join points indicating the percentage of parasitization in two organs from the same patient.

The difference between the sizes of parasites and erythrocytes means that not all sections through a PRBC will include part of the parasite. Because parasite size and shape change during development, it is not practicable to make an accurate estimate of the correction factor needed. Ratios of parasite/RBC diameters were measured on 12 randomly selected electron micrographs. The mean ratio was 0.56, and only two cells had ratios of over 0.7. This would suggest that at least 30% of sections through parasitized cells would not include the parasite and that therefore the frequency of parasitization in some cerebral vessels might approach 70%.

In all cases where samples were available, the order of the degree of parasitization was brain > heart > liver, lung, kidney >> blood. It was apparent that some PRBCs in the liver were within Kupffer cells, but the proportion could not be determined (Figure 12A) because of the small sample size.

The great majority of PRBCs in the kidney were in glomeruli, where some were clearly adherent to glomerular endothelium (Figure 12B). Very few PRBCs were seen elsewhere in the kidney. There were no other significant abnormalities in the glomeruli.

Few specimens of lung which included alveoli were available. In the one case in which a detailed comparison with brain was possible, some interesting differences were apparent. Thus, some alveolar blood vessels contained PRBCs, but the parasites appeared to be almost all in ring or early trophozoite stages (Figure 13A). Other vessels contained large numbers of inflammatory cells, including neutrophils, monocytes, and lymphocytes (Figure 13B). In the example shown, there was no clinical evidence for aspiration pneumonia. On the corresponding brain specimens, the majority of parasites

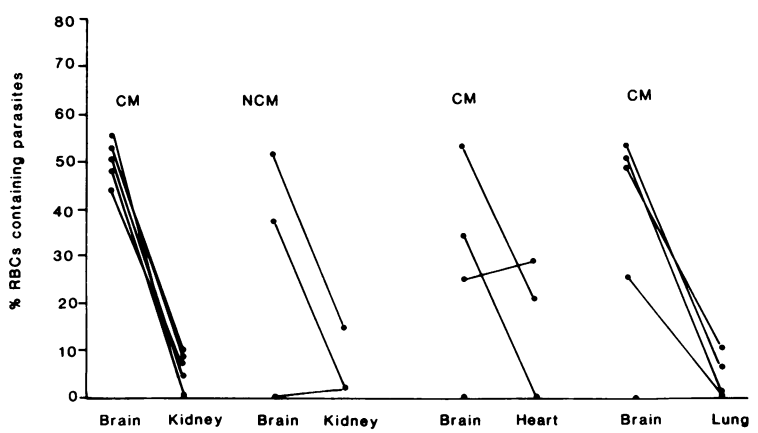

Figure 11—Comparison of the frequencies of parasitized RBCs in different organs from individual patients. The percentage of parasitized RBCs within vessels in different organs was counted for individual patients. The points represent these percentages. The lines join points obtained from different organs in the same patient.

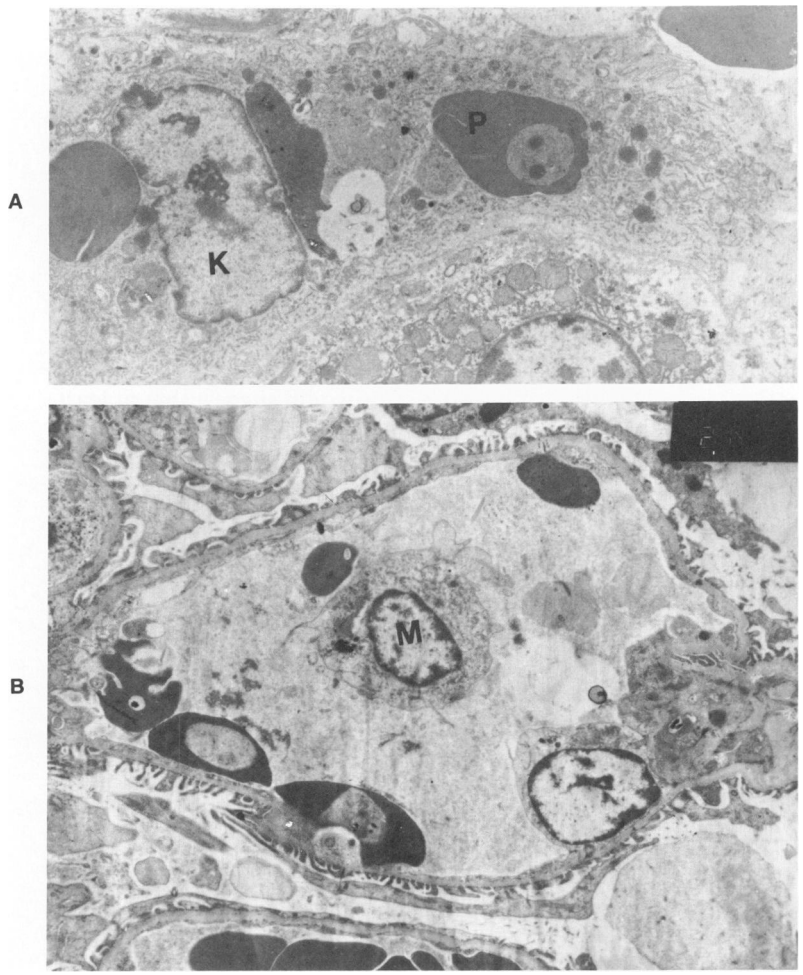

Figure 12A—Noncerebral malaria. Liver. A Kupffer cell (*K*) contains several RBCs, of which some are parasitized (*P*). (×3600) **B**—Cerebral malaria. Kidney glomerulus. Several parasitized RBCs are present and appear adherent to the glomerular endothelium. A monocyte (*M*) is present in the lumen. (×2600)

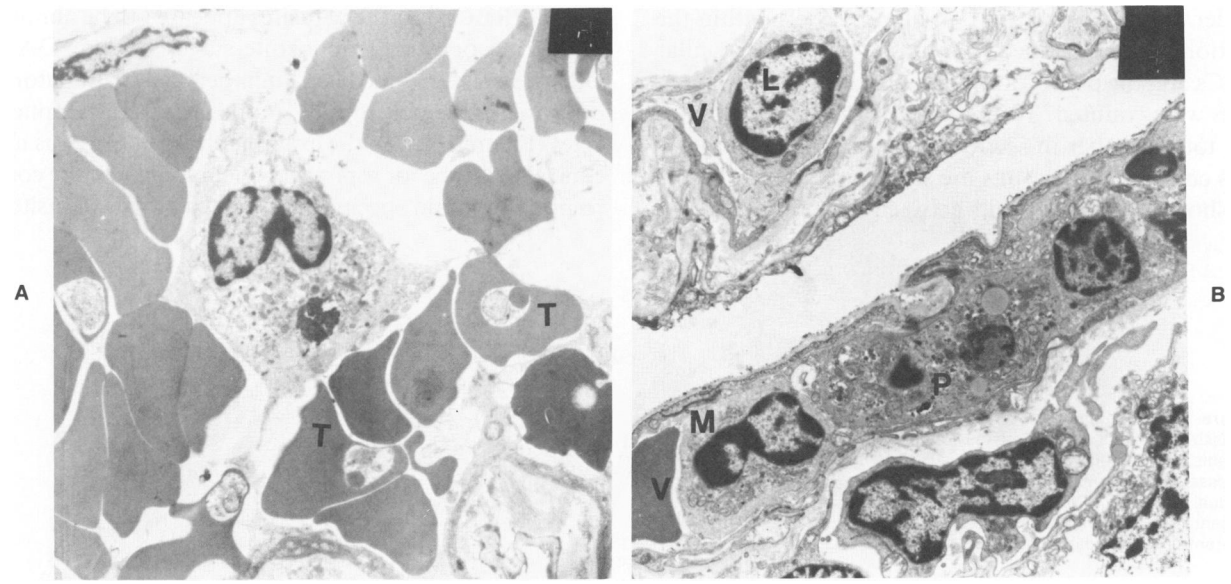

Figure 13A—Cerebral malaria. Lung. Parasitized RBCs are frequent but do not display conspicuous surface knobs. The parasites are relatively immature; ring forms or early trophozoites (*T*). Compare this figure with Figure 15, from the same patient. (×3600) **B**—Cerebral malaria. Lung. Same patient as previous figure. Venules (*V*) are packed with large numbers of inflammatory cells, including neutrophil polymorphs (*P*), monocytes (*M*), and lymphocytes (*L*). (×4600)

were late trophozoites or schizonts, PRBC "ghosts" were present, and no leukocytes were seen.

Three out of the four specimens of heart examined showed intravascular PRBCs. However, although the frequency of PRBCs was quite high (average, 20.1%; range, 21–28%), the vessels did not appear to be tightly packed, and there were no signs of endothelial damage (Figure 14).

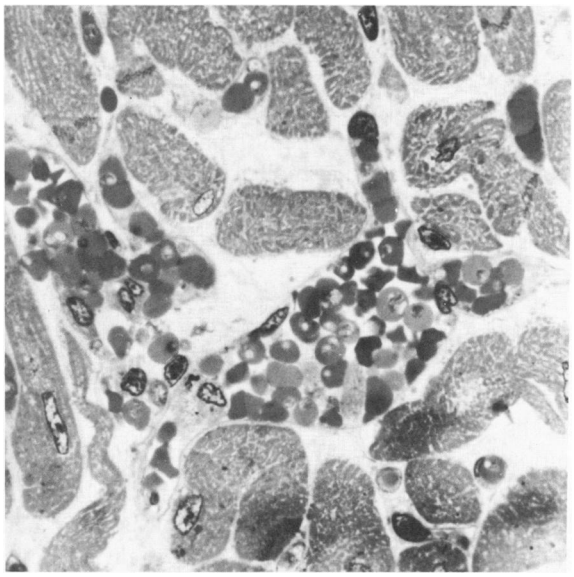

Figure 14—Cerebral malaria. Heart. Light micrograph of a 1-μ section. Blood vessels are filled with RBCs, many of which are parasitized, but the vessel is not tightly packed.

Sequestration of PRBCs in the Skin

The results are given in Table 4. Parasite counts obtained from venous and capillary blood were lower than those from the skin biopsy or skin smear specimens. In the cerebral malaria patients the differences between venous blood and skin biopsy were significant (P = 0.04) and in the uncomplicated malaria group the difference between the capillary sample and the skin smear counts were significantly different (P = 0.03).

Overall comparison of mean capillary and venous parasite counts with the corresponding biopsy and smear counts indicate that the latter samples gave parasite counts 25% higher (mean venous + capillary ± SD, 24.5 ± 23.3, compared with 30.7 ± 30.5 parasitised cells per 1000 cells from the mean biopsy and smear samples; P = 0.005).

Discussion

The aims of the present studies were to correlate the pathologic changes in immediate postmortem specimens from patients dying during the course of *P falciparum* malaria with the clinical syndrome defined as cerebral malaria. Because ultrastructural studies of human CM have not been reported previously, some other observations have also been included.

The characteristic pathologic features of cerebral malaria have been described by several authors.[14] Those most frequently reported are small petechial (ring) hemorrhages, concentrated in the white matter of the cerebral hemispheres, the presence of PRBCs in capillaries and small venules with apparent blockage of vessels by PRBC, and cerebral edema.

Studies carried out on material obtained at routine postmortem are difficult to interpret, because postmortem degeneration in the central nervous system is very rapid, because there is often a lack of control patients infected with *P falciparum* but not showing cerebral symptoms, and because the appearances found at postmortem represent the end stage of processes which have evolved over several days and which have been compounded by the hypoxia, hypotension, and acidosis of the agonal phase.

In this study specimens were taken and fixed immediately after death and indeed showed few signs of postmortem degeneration, a control group of patients was included, and the specimens were examined without knowledge of the clinical diagnosis.

Cerebral malaria was strictly defined as unrousable coma in *P falciparum* malaria after other causes of coma were excluded. A difficulty which cannot be overcome is that at least two patients in the NCM group (Patients 9 and 11) may have had or been about to develop CM but died of other causes before the clinical criteria for this diagnosis were fulfilled. These two patients were in coma but were also hypoglycemic. The methods utilized in this study create another potential problem, that of the small size of the samples. Where possible, several samples were taken from different sites in a single brain, and all samples were included in the analysis. Intersample variation was generally small.

The first conclusion to be drawn from these studies

Table 4—Frequency of Parasitized RBCs in Blood and Skin (% ± 1 SD)

	Venous	Capillary	Skin smear	Skin biopsy
Cerebral malaria (n = 9)	1.63 ± 1.96	1.59 ± 1.52	1.75 ± 2.43	2.03 ± 2.38
Uncomplicated malaria (n = 8)	3.09 ± 2.06	3.89 ± 3.22	4.63 ± 3.87	4.46 ± 3.12

is that there is little evidence of extravascular pathology, either hemorrhagic or inflammatory. Few extravascular RBCs were seen, and some were present in the NCM group. No areas of hemorrhage compatible with those described as "ring" hemorrhages were observed, probably because of the small size of samples and because ring hemorrhages are most common in white matter, whereas the majority of samples we examined came from gray matter.

Extensive destruction of the cerebral microvasculature is also unlikely on clinical grounds; survivors of cerebral malaria make a complete recovery without evidence of neurologic deficit in more than 95% of cases, red cells are not found in the cerebrospinal fluid, and computed tomography of the brain is usually normal.[23]

Leukocytes were seen very rarely outside blood vessels in CM and NCM samples. Some, mainly neutrophils, were seen within vessels, but their frequency did not appear to be increased above normal. The accumulation of mononuclear cells, variously called neuroglia, microglia, or macrophages, described in cerebral malaria[24,25] was not observed; but such accumulations appear to be related mainly to areas of hemorrhage and may represent a secondary phenomenon.

The absence of granular deposits along basement membranes of cerebral venules and of other evidence of vasculitis, together with the absense of inflammatory cells, suggests that immune complexes are not present in the cerebral vessels of CM patients. Immunoperoxidase staining of protease-digested formalin-fixed brain sections from 10 cerebral malaria patients revealed no IgG or IgM deposition (M. J. Warrell, unpublished observations). The absence of any clinical evidence of glomerulonephritis and the finding that serum complement levels are normal except in the most seriously ill patients[26] (S. Tharavanij and P. Malasit, unpublished observations) also argue against an immune-complex-mediated etiology.

We conclude that there is no evidence for either acute or chronic inflammation in human cerebral malaria, and that it is most unlikely that any immune process is involved.

These data contrast strongly with those seen in some rodent malarias,[13] where monocyte accumulation and emigration are conspicuous features of the early stages of the disease. This suggests that the pathogenesis of nervous system involvement in such models differs from that in humans and that there is no satisfactory animal model for human cerebral malaria.

The essential structural components of thrombi are platelets and fibrin,[27] but platelets were strikingly absent from cerebral vessels in CM and NCM patients. In the one example of a breached vessel seen, there were no platelets present. This correlates with the thrombocytopenia described in *P falciparum* malaria.[26] Striated fibrillar deposits, probably of fibrin, were present in a small proportion of cerebral vessels from both CM and NCM patients, but in general, deposits were not seen in vessels with 3+ packing of RBCs, suggesting that thrombosis plays no part in the etiology of CM. Widespread thrombosis of small vessels would also not be compatible with the complete neurologic recovery seen in most CM patients.

Overt endothelial damage was present in a proportion of vessels from all CM specimens, and also in some vessels from some non-CM specimens. In 2 CM cases (Patients 1 and 4) the overall morphology of the brain tissue suggested postmortem degeneration, and almost all vessels were affected. In the others, only a proportion of vessels were damaged, and adjacent vessels had apparently normal endothelium. Because this pattern was observed in both CM and non-CM cases, it is difficult to relate it to the etiology of CM.

The strongest correlation between vascular pathology and CM in the present study is the tightness of packing of RBCs in capillaries and venules, where it is quite clear that CM patients have a higher average density of packing and a considerably larger proportion of tightly packed vessels. It is difficult to account for this degree of packing and for the higher proportion of PRBCs seen in CM, compared with NCM, patients, together with the striking differences in the frequency of PRBCs between the brain and other organs, unless it is due to selective adhesion of PRBCs to cerebral endothelium. Leakage of blood plasma from small vessels in amounts large enough to account for the tight packing might be expected to cause cerebral edema, which is apparently absent except as an agonal phenomenon[23] and would be expected to occur in other organs, without barriers to diffusion, to at least as great an extent as in the brain.

Decreased deformability of PRBCs[8] might similarly be predicted to lead to their accumulation equally in vessels of all organs, and to their retention in capillaries and upstream vessels, rather than in venules.

We conclude that selective adhesion of PRBCs to cerebral vascular endothelium is the most probable explanation.

It is widely accepted that the "knobs" on parasitized RBCs may be involved in adhesion to endothelial cells,[9,10] and studies of the binding of infected RBCs to cultured human endothelial cells[34] and amelanotic melanoma cells[35] strongly suggest that knobs form the sites of attachment of sequestered RBCs, but it is of-

ten difficult to be certain that the morphologic changes described at regions of RBC/endothelial cell apposition, where the radius of curvature of the cells is locally decreased, are not due to local tangential sectioning resulting in the loss of the appearance of normal membrane relationships. The observations in the present study of strands of electron-dense material running preferentially between "knobs" and endothelial membranes do provide concrete evidence that the knobs are indeed related to adhesion *"in vivo."*

For adhesion to occur, PRBCs need to come into contact with endothelium. A striking and unexpected observation was the presence of numerous endothelial pseudopodia in cerebral vessels of both CM and NCM patients, many in intimate contact with and apparently adherent to PRBCs. Similar pseudopodia were not seen in control brain sections or in blood vessels from other organs in CM and NCM patients.

Clark[29] has suggested that cerebral malaria may be caused by increased vascular permeability due to endothelial damage, which may be caused by active oxygen metabolites such as hydrogen peroxide, superoxide anion, and the hydroxyl radical and that one manifestation of endothelial damage is the development of pseudopodia. Such metabolites can be released by neutrophils and by activated macrophages,[30] but there is a conspicuous absence of these cells in the brains of CM patients. Although it is known that some malarial parasites can generate H_2O_2, production is inhibited at low oxygen tensions,[31] and there is no evidence at present that they can produce other oxygen metabolites. In addition, the endothelial pseudopodia described by Clark differ morphologically from those observed in the present study.

Cerebral endothelial pseudopodia have been described in at least two other situations. Yu et al[32] fed chickens on a diet high in linoleic acid and deficient in vitamin E and observed flaplike pseudopodia in cerebral vessels. Schmahl et al[33] induced endotoxin shock in cats and observed microvilluslike structures developing from cerebral vessels, whereas vessels in other organs showed overt endothelial damage.

The development of pseudopodia by cerebral endothelium may be peculiar to these vessels and represent a nonspecific response to injurious stimuli. The greater endothelial surface area available for PRBC contact in cerebral vessels because of pseudopodia might explain the higher frequency of PRBCs in these vessels. It is also possible that plasma membrane specialization in cerebral endothelium leads to the specificity of interactions, but in the absence of cultured endothelium from the brain this hypothesis is difficult to test. The expression of binding molecules by PRBCs could be dependent on the availability of anabolic substrates such as purines and glucose, and purines may be more readily available in hypoxic cerebral capillaries.

Thus, we suggest that the high degree of red cell parasitization seen in the cerebral capillaries results from selective accumulation of PRBCs within these vessels due to the increased adhesiveness of PRBCs for cerebral endothelial cells, perhaps related to the presence of endothelial pseudopodia, and that the neurologic syndrome results from hypoxia and other biochemical disturbances caused by mechanical obstruction of the vessels by cells containing actively metabolizing parasites. Immune or inflammatory processes appear to play no part in the pathogenesis of the syndrome.

Other Observations

The mechanism by which parasite proteins are transported to the plasma membrane of the RBC is not clear. The observation of parasite-associated membranes within the cytoplasm of PRBCs and the presence of electron-dense material between these membranes and the plasma membrane suggests that they may possibly be involved in transport of material from the parasite to the plasma membrane of the cell, but morphologic observations alone cannot provide decisive evidence.

The term "crisis form" describes degenerative changes in intraerythrocytic parasites at the time of crisis in monkey malarias, possibly caused by tumor necrosis factor or free oxygen radicals.[38] In this study a proportion of PRBCs within cerebral vessels contained parasites showing morphologic signs of damage similar to crisis forms. In the absence of local inflammatory cells able to release mediators, it is possible that the parasites were damaged elsewhere in the circulation before becoming arrested in the cerebral vessels.

Our observation that parasite counts were higher in skin smears or biopsies than in peripheral blood samples lends some support to the claim by Chinese workers that blood sampled by intradermal skin pricks is a more sensitive method of diagnosing *P falciparum* malaria than the conventional peripheral blood smear or even bone-marrow aspiration.[39]

Conclusions

1) The clinical syndrome of cerebral malaria correlates closely with the presence of tight packing of cerebral venules and capillaries by PRBCs. In the absence of thrombosis or inflammation, the most likely cause

is selective adhesion of PRBCs to cerebral endothelial cells.

2) There is a selective accumulation of PRBCs in cerebral vessels, as compared with vessels in other organs examined. Sequestration is least apparent in the skin.

3) There is morphologic evidence of adhesion of PRBCs to cerebral endothelium via their surface knobs.

4) There is no evidence that overt endothelial damage plays an important part in this condition, although the presence of endothelial pseudopodia may be related to the selective accumulation of PRBCs in the brain.

5) There may be no need to involve factors other than metabolic disturbances consequent upon a reduction in cerebral microcirculatory flow to account for the clinical features of CM.

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Acknowledgments

We are extremely grateful for the excellent technical assistance of Mrs. Kamolrat Silamut in Bangkok and Ms. M. Bergin-Cartwright and Mr. C. Jenkins in Oxford. Mrs. P. R. Woodward typed the manuscript both accurately and quickly. Dr. F. H. C. Marriott, Department of Biomathematics, helped with the statistical analysis of data.