

A Quantitative Analysis of the Microvascular Sequestration of Malaria Parasites in the Human Brain

Kamolrat Silamut,* Nguyen H. Phu,[†]
Christopher Whitty,[‡] Gareth D.H. Turner,^{‡§}
Karina Louwrier,[§] Nguyen T.H. Mai,[†]
Julie A. Simpson,^{*‡} Tran T. Hien,[†] and
Nicholas J. White^{*‡}

From the Faculty of Tropical Medicine,* Mahidol University, Bangkok, Thailand; the Center for Tropical Diseases,[†] Cho Quan Hospital, Ho Chi Minh City, Viet Nam; The Oxford-Wellcome Centre for Tropical Diseases,[‡] Nuffield Department of Clinical Medicine, University of Oxford, and the Department of Clinical Biochemistry and Cellular Science,[§] University of Oxford, United Kingdom

Microvascular sequestration was assessed in the brains of 50 Thai and Vietnamese patients who died from severe malaria (*Plasmodium falciparum*, 49; *P. vivax*, 1). Malaria parasites were sequestered in 46 cases; in 3 intravascular malaria pigment but no parasites were evident; and in the *P. vivax* case there was no sequestration. Cerebrovascular endothelial expression of the putative cytoadherence receptors ICAM-1, VCAM-1, E-selectin, and chondroitin sulfate and also HLA class II was increased. The median (range) ratio of cerebral to peripheral blood parasitemia was 40 (1.8 to 1500). Within the same brain different vessels had discrete but different populations of parasites, indicating that the adhesion characteristics of cerebrovascular endothelium change asynchronously during malaria and also that significant recirculation of parasitized erythrocytes following sequestration is unlikely. The median (range) ratio of schizonts to trophozoites (0.15:1; 0.0 to 11.7) was significantly lower than predicted from the parasite life cycle ($P < 0.001$). Antimalarial treatment arrests development at the trophozoite stages which remain sequestered in the brain. There were significantly more ring form parasites (age < 26 hours) in the cerebral microvasculature (median range: 19%; 0–90%) than expected from free mixing of these cells in the systemic circulation (median range ring parasitemia: 1.8%; 0–36.2%). All developmental stages of *P. falciparum* are sequestered in the brain in severe malaria. (*Am J Pathol* 1999, 155:395–410)

Severe falciparum malaria remains one of the most important causes of death in the tropics. Cerebral malaria is the major lethal manifestation of this infection. The sequestration of red blood cells containing mature forms of *Plasmodium falciparum* in the cerebral microvasculature is considered to be the essential underlying pathological process, although how this leads to coma and death remains unresolved.^{1–4} Sequestration results from the adherence of parasitized red blood cells to vascular endothelium.⁵ *In vitro* studies suggest that infected red blood cells begin to cytoadhere at the late ring or early trophozoite stage of parasite development.⁶ Studies *in vivo* and histopathological observations in fatal *P. falciparum* malaria and also in the lethal sequestering animal malarias suggest that the red cells containing more mature parasites are sequestered in the brain and other organs from the middle of the parasite life cycle until schizont rupture.^{7–16} The next generation of motile daughter merozoites is then released and invades more red cells to continue the blood stage infection. In most patients with falciparum malaria relatively few *P. falciparum* trophozoites and schizonts are seen in the peripheral blood, while these more mature parasites are abundant in the capillaries and venules of vital organs (particularly the brain).¹⁰ The kinetic processes involved and the microvascular distribution of sequestration have not been characterized previously. Post-mortem studies have given conflicting impressions on the predominant stage of parasite development encountered in the brain. MacPherson et al¹⁰ found that both trophozoites and schizonts predominated, whereas Lemercier et al¹⁷ reported almost exclusively schizonts. Others have reported that all stages of parasite development are represented.^{7,8,11} The aims of this study were to describe the microvasculature pattern and distribution of parasite developmental stages in the brain in fatal cases of severe falciparum malaria.

Supported by The Wellcome Trust of Great Britain through the Wellcome-Mahidol University- Oxford Tropical Medicine Research Programme in Thailand, and the Wellcome Trust Clinical Research Unit, Center for Tropical Diseases in Vietnam.

Accepted for publication April 11, 1999.

Address reprint requests to Prof. Nicholas J. White, Faculty of Tropical Medicine, 420/6 Rajvithi Road, Bangkok 10400, Thailand. E-mail: fnnjw@diamond.mahidol.ac.th.

Methods

These studies were conducted at the Center for Tropical Diseases, Cho Quan Hospital, Ho Chi Minh City, Viet Nam, and Paholpolpayahasena Hospital, Kanchanaburi, Thailand. All patients were admitted with severe falciparum malaria and were treated with either intravenous quinine dihydrochloride (20 mg salt/kg over 4 hours, followed by 10 mg/kg every 8 hours), intramuscular quinine (same dose regimen), or intramuscular artemether (4 mg/kg followed by 2 mg/kg every 8 hours). All died from direct complications of their illnesses. Most of the patients in Vietnam were included in a prospective double-blind comparison of artemether and quinine which has been reported elsewhere.¹⁸ The pathological assessments in these patients were made before unblinding the study.

Procedures

Post-mortem sampling was performed only after fully informed permission was granted from attendant relatives. Where a full autopsy was performed (in Vietnam only), these samples were taken after removal of the brain. In six cases samples were examined from three sites: medulla, cerebellum and cortex. When an autopsy was not performed samples were taken using a Vim-Silverman needle inserted through the superior orbital foramen or the foramen magnum. For this study the specimens were not sectioned, but smeared by placing the brain specimen between two glass microscope slides, pressing these together, and making thin smears.¹⁹ This method preserves long fragments of capillaries and venules. Brain biopsies were performed within 2 hours of death, and autopsies within 6 hours. Slides were fixed in absolute methanol and stained by the reverse Field's method.²⁰ Malaria parasites and pigment were easily identified. Intravascular parasites were counted and staged. Peripheral blood slides were prepared either from blood taken post-mortem (by ventricular puncture) or, if an ante-mortem blood smear had been taken within 4 hours before death, this was used.

Morphological Assessment of Parasite Stage of Development

In Vitro Study

In order to validate the subsequent histopathological assessment of parasite stage of development, *in vitro* cultures two of different isolates of *P. falciparum* were synchronized carefully by repeated exposure to sorbitol until a 1-hour time window was obtained. Smears were then taken throughout one asexual life cycle (every 2 hours until 42 hours, then every 1 hour). The parasite morphology at precise times before the midpoint of schizogony *in vitro* (see Figure 1) formed the basis of the assessment of parasite development stages in the cerebral vessels. The effects of IC₉₉ concentrations of quinine and artemether on parasite morphology were assessed

from *in vitro* cultures of parasite isolates with predetermined antimalarial susceptibility.

In Vivo Studies

Within the cerebral vessels the red cells and the malaria parasites were compressed within the confines of the packed sequestered capillaries or venules. The diameter of a capillary is smaller than that of an erythrocyte, and red cells have to bend and deform in their passage through the microvasculature. The separation between red cells in brain smears was often difficult to determine, but the individual parasite nuclei and cytoplasm and malaria pigment were readily identified. In order to assess the effects of red cell compression within narrow vessels on parasite morphology, simulation experiments were conducted in the rat. The midpoint capillary diameters in the rat are smaller than those in humans, and so distortion should have been greater in the experiments than in the clinical samples. Large volume *in vitro* *P. falciparum* cultures (5 ml of blood at 15% hematocrit and 10–30% parasitemia) were prepared and infused via a 26-gauge catheter inserted into the aortic arch of anesthetized (6% chloral hydrate given by intraperitoneal injection) male Wistar rats. The inferior vena cava and descending aorta were clamped, and an equal volume of blood removed as that infused. The objective was to fill the cerebral vessels with human blood from the parasite culture. As the infusion was finished, cardiac arrest was induced. The brain was removed and smears from gray matter prepared in exactly the same way as the post-mortem slides were prepared in the clinical study. *P. falciparum* infected red cells at different stages of development in the cerebral microvasculature of the rat in brain smears were compared with those in heart blood smears.

Parasite Staging and Counting

In the brain smear from each case of fatal falciparum malaria a minimum of 5 capillaries were selected randomly, the parasites counted, and at least 10 examined for stage of development. A minimum of 20 sequential red cells in each capillary were examined. Discrete sections of capillaries or venules containing less than 20 red cells were not assessed. Eight stages of parasite development (approximate parasite age) were recorded: tiny (0–6 hours) small (6–16 hours), and large rings (16–26 hours); early (26–30 hours), middle (30–34 hours), and late trophozoites (34–38 hours) and schizonts with up to (38–44 hours) or more than five nuclei (44–48 hours) (Figure 1). A total of 100 parasites were staged. The distribution of malaria pigment in each vessel was also noted, and any white cells or gametocytes were recorded. Thin film peripheral blood smears on admission and before death were examined in the same way for parasite count and stage of development assessment. Each brain and blood smear was examined independently by two people, and the smears re-read if there was significant disagreement.

Immunohistochemical Staining

Brain tissue from autopsy cases was snap-frozen in liquid nitrogen. Tissues were either taken immediately post-mortem by blind needle biopsy or at open autopsy. Control tissues were taken at autopsy in the United Kingdom from cases of sudden death excluding patients who died from systemic sepsis, central nervous system disease, or ischemia. Small tissue fragments were either cut directly from the biopsy cores with a scalpel blade or a 20- μ m section cut on a cryostat. These were then smeared onto Vectabond-coated slides, air dried, and fixed in 100% acetone for 10 minutes. Slides were immunostained immediately using a standard indirect immunoperoxidase technique. The monoclonal antibodies used for recognition of the host receptor antigens and constitutive control antigens were as follows: class I HLA = W6/32; class II HLA = C3/43; CD31 = JC70A; VCAM-1 = 1.4C3; E-selectin = 1.2B6 (DAKO, UK); chondroitin sulfate = CS-56 (reacts to both CS-A and CS-C) (Sigma, UK); ICAM-1 = 15.2 (gift of Dr. N. Hogg); CD36 = ES IVC-7 (gift of Dr. E. Van der Schoot, Amsterdam); thrombospondin = 189/53.2 (gift of Dr. J. Dawes, Edinburgh); P-selectin = RUU-SP 2.15.1 (gift of Dr. R. Niewenhuis, Utrecht). All antibodies were either used as supernatants or reconstituted from lyophilized ascites in PBS/0.1% BSA and stored at 4°C. The monoclonal primary antibody was added to the sections for 30 minutes at room temperature in a moist chamber. Slides were then washed three times in PBS before adding the second layer of peroxidase conjugated goat anti-mouse immunoglobulins (DAKO P0447) at 1:50 dilution in PBS/0.1% BSA. The solution was removed after 30 minutes of incubation at room temperature, washed in PBS three times, and then stained with the chromogen fast DAB tablets in PBS (Sigma, UK). After incubating for 8 minutes the sections were then washed in tap water, counterstained with hematoxylin and mounted with Aquamount.

Slides were examined and graded blindly by two independent observers (K.L. and G.T.). The degree of staining and the number of vessels showing staining were assessed using a semi-quantitative score: -, negative (no endothelial cell staining); +/-, scattered endothelial cells positive on <25% vessels; +, positive endothelial cell staining on >25% and <80% vessels; ++, strong positive endothelial staining on >80% vessels. The percentage of cases and intensity of staining was then compared between control and malaria cases.

Statistical Analysis

The data from these analyses were not normally distributed and nonparametric methods were used for all statistical analyses. The Wilcoxon signed rank test was used to compare brain and peripheral blood results. The Mann-Whitney *U* test was performed when comparing the effects of the different antimalarial treatments and the Spearman rank correlation coefficient measured associations with time from treatment to death. To determine if the age of parasites differed across brain capil-

laries the mean and standard deviation of all parasites pooled within a subject were calculated. Using these distributions five datasets of 10 values were randomly simulated for each subject. The standard deviation of the simulated means was compared to the standard deviation of the observed means (for each brain capillary) using the Wilcoxon signed rank test. The level of significance was 5% and all analyses were performed using the statistical package SPSS for Windows (SPSS Inc.).

Results

Clinical Findings

Brain smears were obtained from 50 Vietnamese and Thai patients who died from complications related to severe malaria (49 *P. falciparum*, 1 *P. vivax*) between 1986 and 1994. There were 13 women (age range 16–51) and 37 men (age range 13–67). The mean age was 32 years. Cerebral malaria was the main complication in 20 patients, while 12 had both cerebral malaria and renal failure, and 8 had renal failure without cerebral symptoms. Pulmonary edema was the principle cause of death in 6 cases. One patient died from algid malaria, one from lactic acidosis without cerebral involvement, and one from supervening staphylococcal septicemia. The patient who died with vivax malaria was a 40-year-old woman who was admitted fully conscious but with severe anemia (hematocrit 7%), jaundice, and hypoglycemia (plasma glucose 2.0 mmol/L). The *P. vivax* parasite count was 0.6%. She was treated with intravenous artesunate as coincident *P. falciparum* could not be excluded with confidence and was transfused with blood. Parasite clearance time was 48 hours. On the third day of admission she died suddenly and could not be resuscitated. Of the other 49 patients who died from falciparum malaria, 16 had hyperparasitemia on admission (parasitemia >10%).

The mean duration of preceding disease was 6.5 days (range 3–11). Elective treatment with intravenous quinine alone was given in 18 cases and artemether in 1 case; the other patients were randomized to receive either intramuscular quinine (18 cases) or artemether (12 cases) in the double blind trial.¹⁸ Before admission to hospital 23 patients had received quinine, 2 artesunate, 2 pyrimethamine-sulphadoxine, 1 mefloquine, and 1 chloroquine treatment. The median duration of antimalarial treatment before death was 2 days (range 1.5 hours to 7 days).

Pathological Findings

Complete data were available on the parasite count per 100 red blood cells for the brain and per 1000 red cells for blood for 46 of the 50 cases studied. Four were excluded from the quantitative analysis (3 *P. falciparum*, 1 *P. vivax*). The three patients with falciparum malaria died from renal failure. Each had cleared the peripheral parasitemia and no parasites were found in the brain, although significant amounts of malaria pigment were still evident in the small vessels, indicating that sequestration

0-6 H	6-16 H	16-26 H	26-30 H	30-34 H	34-38 H	38-44 H	44-48 H
TINY RINGS	SMALL RINGS	LARGE RINGS	EARLY TROPH.	MID TROPH.	LATE TROPH.	SCHIZONTS	SCHIZONTS
width of cytoplasm < 1/2 nucleus	width of cytoplasm ≥ 1/2 nucleus	width of cytoplasm ≥ nucleus	light brown pigment first visible	brown pigment, dark cytoplasm, nucleus and cytoplasm enlarged	becoming spherical, brown pigment, irregular shaped nucleus ≤ 2	dark brown pigment, 3 to 5 nuclei red cell cytoplasm paler	dark brown pigment, > 5 nuclei

Figure 1. Representative parasites (magnification, ×1000) from a highly synchronized *in vitro* culture (initial time window: 1 hour) of *Plasmodium falciparum*. The stage of development is assessed from the overall size of the parasite, the ratios of the area and diameter of the nucleus to that of the cytoplasm, the amount of visible pigment (hemozoin), and, in mature parasites, the number of nuclei.

had occurred at some time. No parasites and no pigment were seen in the brain vessels of the patient who died from vivax malaria.

Sequestration

For the 46 patients with residual malaria parasites, the parasite count in the brain median (range) = 66.5% (1–99) was significantly higher than in the peripheral blood median (range) = 1.4% (<0.1–36.6) ($p < 0.001$). This confirms significant sequestration in the cerebral microvasculature. To investigate whether sequestration in the brain was associated with the duration of or type of antimalarial drug treatment an index reflecting the proportion of sequestered erythrocytes at the time of death was calculated.

$$\text{Sequestration index (SI)} = 10 \times (\text{number of parasitized cells in brain}/100 \text{ red cells}) \div (\text{number of parasitized cells in peripheral blood just before death}/1000 \text{ red cells})$$

For patients where the peripheral blood parasite count was extremely low, the conservative value of 0.05% was chosen in order to calculate SI. The overall median (IQR, range) sequestration index was 40 (9.9–273.8 : 1.8–1500). The median (IQR, range) ratio of the proportion of parasitized red cells in the brain to the admission parasitemia was 15.3 (3.8–41.1: 0.53–1360). There was a significant negative association between time from treatment to death and SI (Spearman correlation coefficient = -0.53 , $p < 0.001$). The median (range) sequestration index for patients treated with quinine was similar to those

who received artemether (41.1 (2.3–1360) versus 39.4 (1.8–1500), $p = 0.91$). The median (IQR, range) SI was not significantly different between those patients admitted with cerebral malaria ($n = 28$), and those who were conscious on admission ($n = 18$); 40 (9.4–299; 1.8–1500) versus 38.5 (9.9–280, 2.3–720), $p = 0.75$. Eleven patients with no clinical history or signs of cerebral malaria had high parasite counts in the brain (more than 50% parasitemia), and in seven of these, over 75% of red cells seen were parasitized, indicating that significant cerebral sequestration had occurred (sequestration index range 3.9–42.2).

Stage of Parasite Development

Assessment in Vitro

The different stages of *P. falciparum* development from the tightly synchronized *in vitro* culture are shown in Figure 1, together with identifying characteristics. The majority of parasites in the peripheral blood smears were immature ring forms, with very few mature trophozoites or schizonts. *In vitro* artemether at IC_{99} concentrations arrested parasite growth and led to altered parasite shape and a significant change in red cell to cytoplasm and nuclear cytoplasm ratios of medium and large rings (Figure 2). Artemether did not affect assessment of the tiny ring stage or the trophozoite or schizont stage assessments based on malaria pigment production and number of nuclei. Quinine at IC_{99} concentrations had much less of an effect on ring form morphology. Although both drugs affected the morphology and staining of the para-

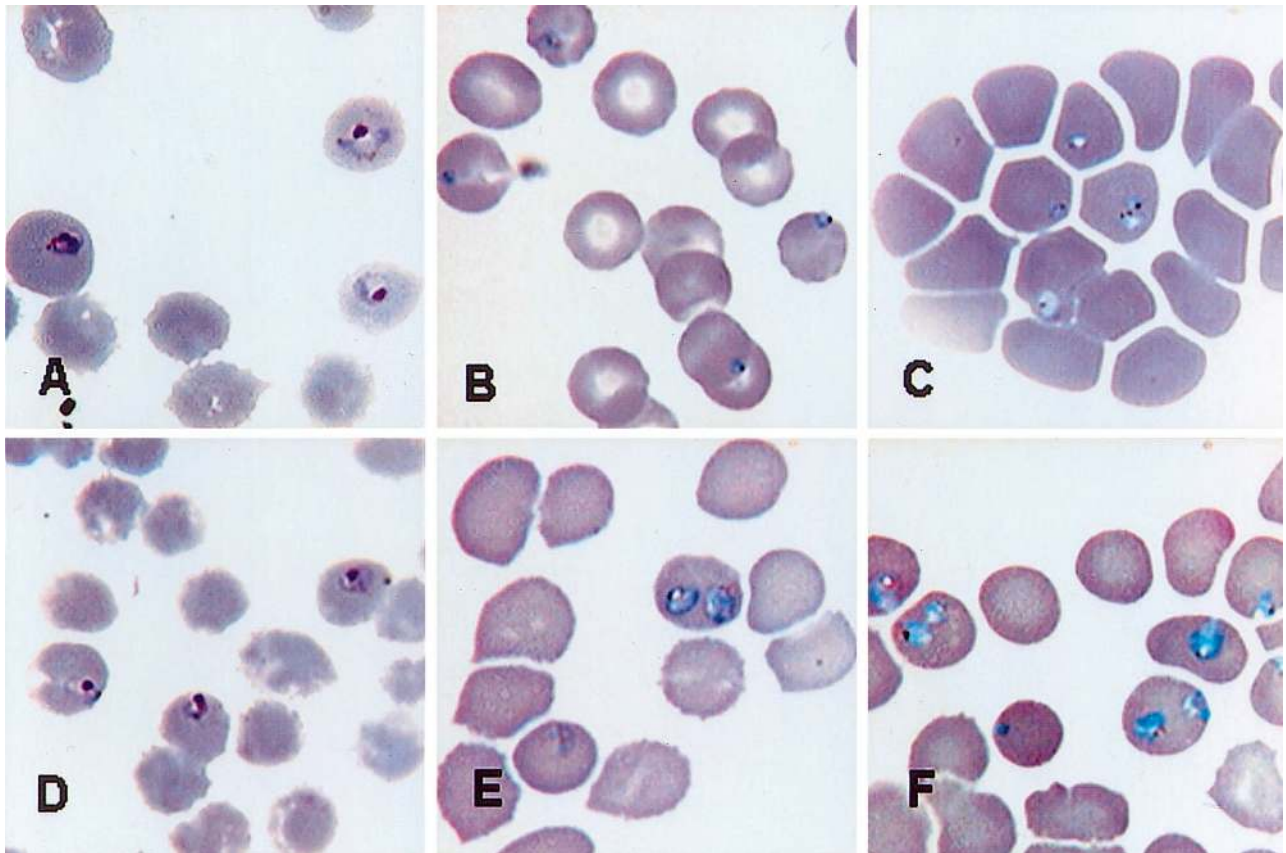


Figure 2. Early trophozoites in *in vitro* culture before exposure (A and D) and then 12 (B and E) and 24 hours (C and F) after exposure to IC₉₉ concentrations of artemether (top row: A–C) and quinine (bottom row: D–F).

site cytoplasm, neither drug affected the overall distribution assessment or caused disappearance of pigment.

Simulation Experiments

In the simulation experiments in 10 rats, the malaria parasites, particularly the younger forms, appeared smaller in the cerebral vessels than in the peripheral (heart) blood. We investigated whether the following relationships were preserved: (a) the relative size of the parasite to the size of the parasitized red cell; (b) the amount of parasite cytoplasm in relation to the nucleus; (c) the amount and distribution of malaria pigment (hemozoin); (d) the number of parasite nuclei.

The size of the parasite in relation to the size of the red cell (a) was distorted and could not be assessed reliably, largely because of the difficulty in identifying reliably the red cell borders. The parasite cytoplasmic dimensions (b) were significantly smaller in the brain vessels, and there was compression of the food vacuole. For example, for parasites at the young ring stage in smears from culture the mean cytoplasmic diameter was 0.67 (0.14) compared with 0.54 (0.08) in the rat brain vessels ($p = 0.002$). However, the ratio of the thickness of the cytoplasm on the opposite side of the food vacuole in relation to the diameter of the nucleus still allowed a stage assessment, and this was not significantly different to that in the heart blood smears; eg small rings 1.86 (0.32) in

culture versus 1.94 (0.33) in the brain ($p = 0.48$). The other relationships were preserved; in particular intra- and extraparasitic malaria pigment (c) was easily identified and quantitated. There was no significant difference between the overall stage distributions assessed in the rat heart blood and rat cerebral vessel smears ($p = 0.26$). These data suggested that stage assessments derived from the *in vitro* experiments could be made in the cerebral vessels in the clinical samples.

Morphological Criteria

The following criteria were used in the assessment of parasites in the brains of the fatal cases. Red cells were identified by their pink (hemoglobin) coloration and, if possible, discrete borders. It was not always possible to distinguish the red cell borders. Malaria parasites were identified from their characteristic staining and intravascular location. Ring stages were identified by their characteristic shape, mature trophozoites by the presence of associated malaria pigment and schizonts by the presence of more than two nuclei. Criteria for distinguishing between merozoites in an intact schizont, a ruptured schizont, and ring forms in newly invaded erythrocytes were established in brain smears from five cases where all were evident. The mean (SD) distance between adjacent merozoites in formed schizonts, choosing the five most separated merozoites, was 0.33 (0.23) ($N = 139$),

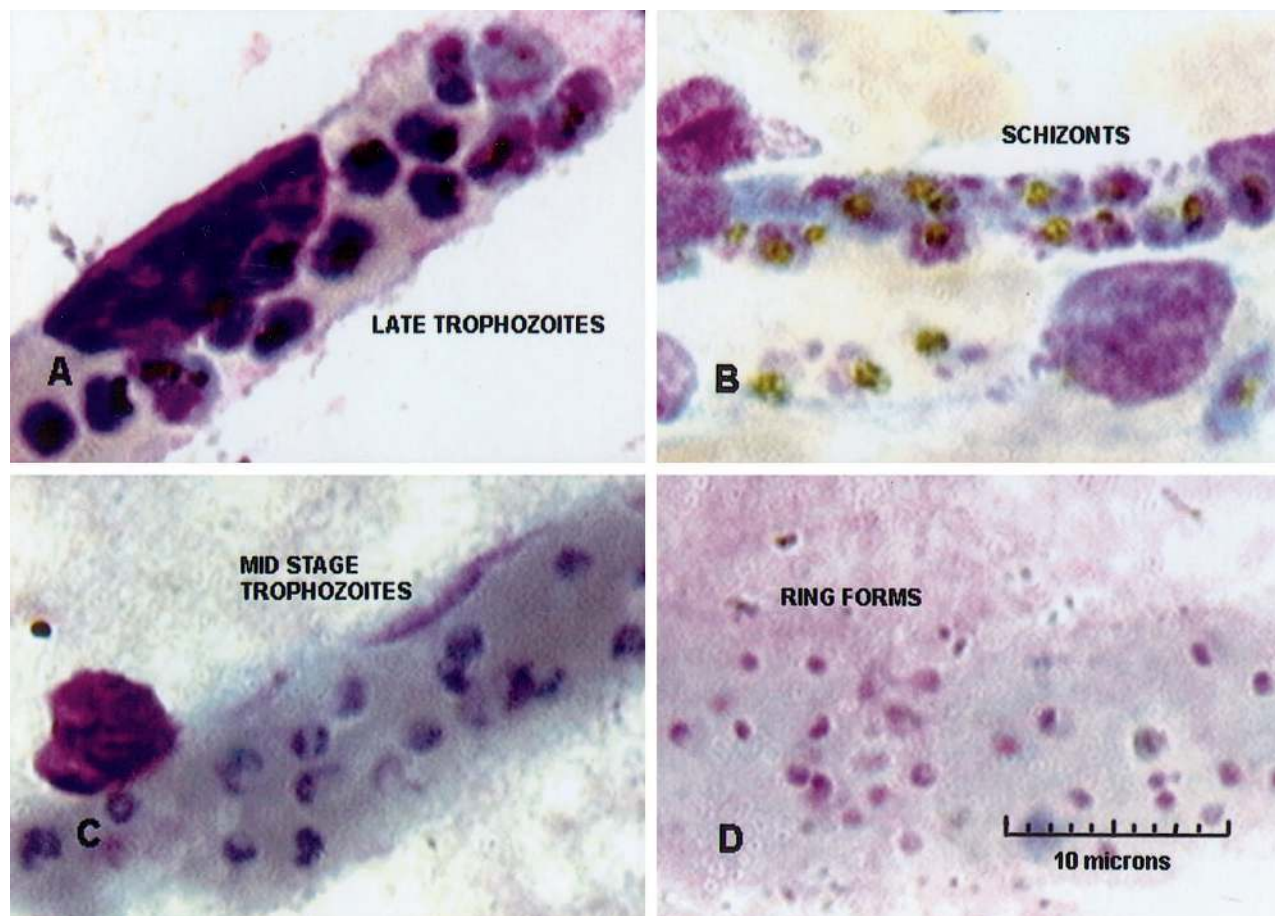


Figure 3. Brain vessels from four different cases of fatal falciparum malaria showing accumulations of different parasite stages. A, late trophozoites, B, schizonts with abundant pigment; C, mid-stage trophozoites; D; ring forms containing no intraerythrocytic pigment.

compared to 0.77 (0.65) μm ($N = 127$) between merozoites liberated from a ruptured schizont, and 3.29 (2.06) μm ($N = 191$) between the parasite nuclei in adjacent ring infected erythrocytes. Thus circular collections of merozoites with an average intermerozoite separation of $<0.5 \mu\text{m}$ were considered intact schizonts, irregular collections of merozoites with an average separation between 0.5 and 1.25 μm were considered ruptured schizonts, and parasite nuclei separated by $>1.25 \mu\text{m}$, apparently surrounded by a red cell, were considered as newly invaded erythrocytes.

Clinical Study

Although all stages of malaria parasite development were seen in these fatal cases, the most frequent parasite form found in the brain was the late trophozoite stage (Figure 3, Table 1). Using the midpoint of the modal stage to represent the parasite age (in hours) for each patient, the brain vessels were found to have parasites that had a modal age approximately 21 hours older than the peripheral blood: median (range) = 32 (3–46) compared with 11 (3–36) hours ($P < 0.001$). The age of parasites in both the brain and the blood was not significantly different between those treated with quinine ($N = 25$) and artemether; ($N = 5$) median (range) for the brain was 32

(3–46) versus 36 (3–46) hours respectively and for the blood was 11 (3–36) versus 21 (3–32) hours, respectively. Peripheral parasitemias in the other patients were too low for accurate assessment of stage distributions. The age of parasites in the brain was correlated negatively with the time from starting treatment to death (Spearman correlation coefficient = -0.42 , $p = 0.004$). There was no relationship between the peripheral blood stage and du-

Table 1. Distribution of the Modal Developmental Stage of *P. falciparum* Parasites in the Peripheral Blood and Brain in Fatal Falciparum Malaria

	Blood* ($n = 30$) N (%)	Brain* ($n = 45$) N (%)
Tiny rings (0–6 h)	8 (26%)	5 (11%)
Small rings (6–16 h)	12 (40%)	3 (7%)
Large rings (16–26 h)	6 (20%)	4 (9%)
Early trophozoites (26–30 h)	0	4 (9%)
Middle trophozoites (30–34 h)	3 (10%)	8 (18%)
Late trophozoites (34–38 h)	1 (3%)	18 (40%)
Schizonts		
5 nuclei (38–44 h)	0	0
>5 nuclei (44–48 h)	0	3 (7%)

*Only for those patients where at least 20 parasites were staged.

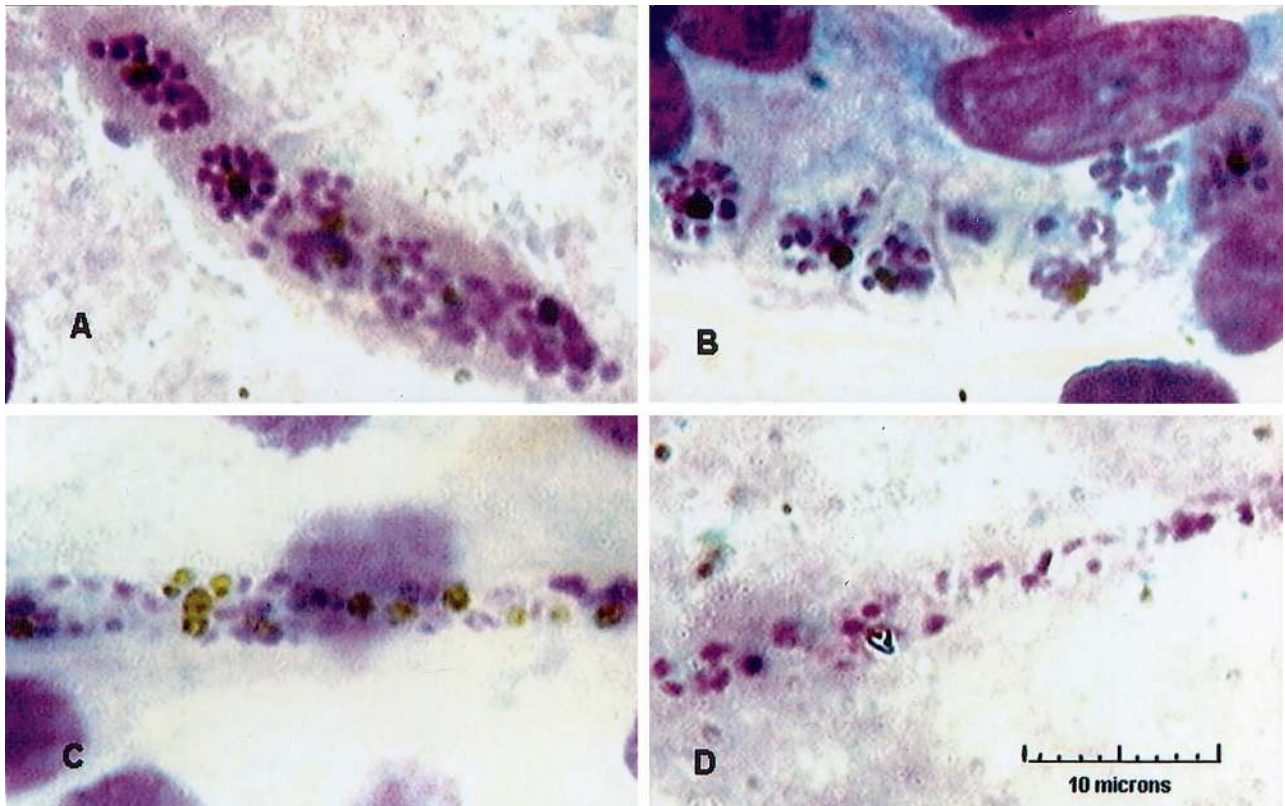


Figure 4. Brain vessels from four different cases of fatal falciparum malaria showing sequestration of intact schizonts (A and B), and free merozoites after schizont rupture (C and D). The intraparasitic malaria pigment has stained black in A and B, and the free pigment in C is colored brown-black.

ration of treatment (Spearman correlation coefficient = 0.11, $p = 0.56$).

Mature Trophozoites and Schizonts

If it is assumed that there is no reason why death should be associated with a particular stage of parasite development, then the distribution of mature stages in the cerebral vessels should be random between patients. To assess this the trophozoite and schizont counts were normalized, by converting each to the number per hour, to adjust for the difference in their respective time spans. Schizonts were often seen (Figure 4) but were significantly under-represented relative to trophozoites in the cerebral microvasculature. The median ratio of schizonts to trophozoites was 0.15 (range, 0.0 to 11.7) and this differed significantly from the expected value of 1 ($p < 0.001$). Examining multiple sites from six brains demonstrated no evidence that this was due to a sampling error. Those brains which had large numbers of schizonts had them in every site sampled, and those that had none in one site had none in others. Thus there were no obvious differences between sites from the same brain.

Ring Form Parasites

It is believed that parasites <26 hours old circulate freely in the bloodstream, whereas parasites >26 hours old are largely stuck in the capillaries and venules. In order to

see whether the proportion of young parasites present in the brain was the same as that which would be expected from the numbers present in circulating blood, the proportion of red cells containing parasites <26 hours old was calculated (for the brain red cells containing parasites >26 hours old were excluded from the denominator). The brain was found to have a considerably higher proportion of red cells containing young parasites compared to peripheral blood: median (range) = 19.0% (0–90%) versus 1.8% (0–36.2%) ($p = 0.001$). All stages of parasites in the first half of the asexual life cycle were over represented (ie this preponderance was not confined to the more mature ring form parasites) (Figure 5 A–C). The proportion of all parasites <26 hours old that were very young rings (aged 0–6 hours) was similar in the brain and peripheral blood; median (range); 21.3% (0–100%) compared with 28.6% (0–94.4%), respectively, ($p = 0.22$). In four of the 50 cases, more than 70% of the parasites in the brain were ring forms.

Microvascular Distribution of Parasite Stages

Individual capillaries within each brain showed very wide variations in the numbers of parasitized red blood cells (PRBC). In several brains some capillaries contained only unparasitized red cells, whilst others in the same section had 100% (all 20/20) of red cells parasitized. Stage of development also varied considerably (Figure 6). For example, one capillary might have 60% of parasites in the

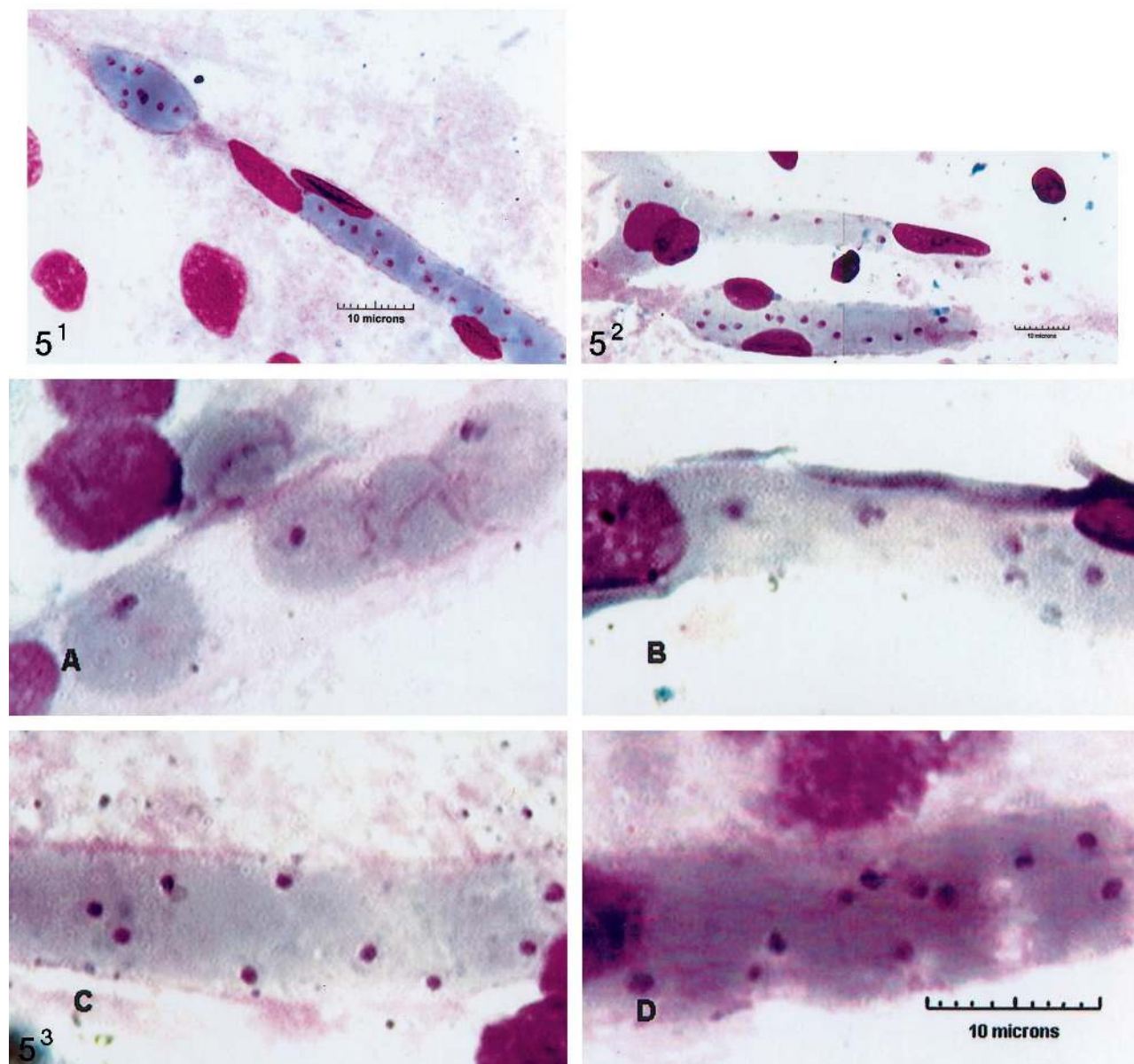


Figure 5. Ring stage parasites in cerebral microvessels. 5^1 : Accumulation of red cells containing ring form parasites in a cerebral vessel. The peripheral parasitemia at death was 0.9%. 5^2 : Composite of two photomicrographs showing accumulation of ring stage parasitized red cells in a cerebral vessel. The peripheral parasite count was 0.2%. Both patients received quinine treatment. 5^3 : Composite of vessels from four different patients (A–D), all of whom received quinine, showing ring stage accumulation.

later ring forms, with no trophozoites, while another capillary from the same brain had 80% late trophozoites with no rings. In some smears where a particularly long vessel was present clustering of different stages could be seen within a single vessel (Figure 7). To see if the age of parasites differed between vessels within a subject, the mean age of parasite development and its standard deviation were calculated for each capillary. These values were then compared with the mean and standard deviation for all the staged parasites (ie the sum of all 5 vessels counted). In order to determine if the individual capillary means and their variances differed from that which would be expected from a single distribution of parasite stages, five datasets of 10 values were simulated from the overall distribution of that subject. The simulated means and

their standard deviations were compared to those observed. There was significantly more variation of parasite age between capillaries than would be expected from a single distribution of parasites; median (range) = 4.12 (0.52 to 15.47) compared to 1.89 (0.58 to 5.71) respectively ($p < 0.001$). Thus each vessel tended to have a single relatively narrow age distribution of sequestered parasites.

Immunohistochemical Staining for Sequestration Receptors

Samples were available from 13 of the fatal cases of falciparum malaria and 10 controls.

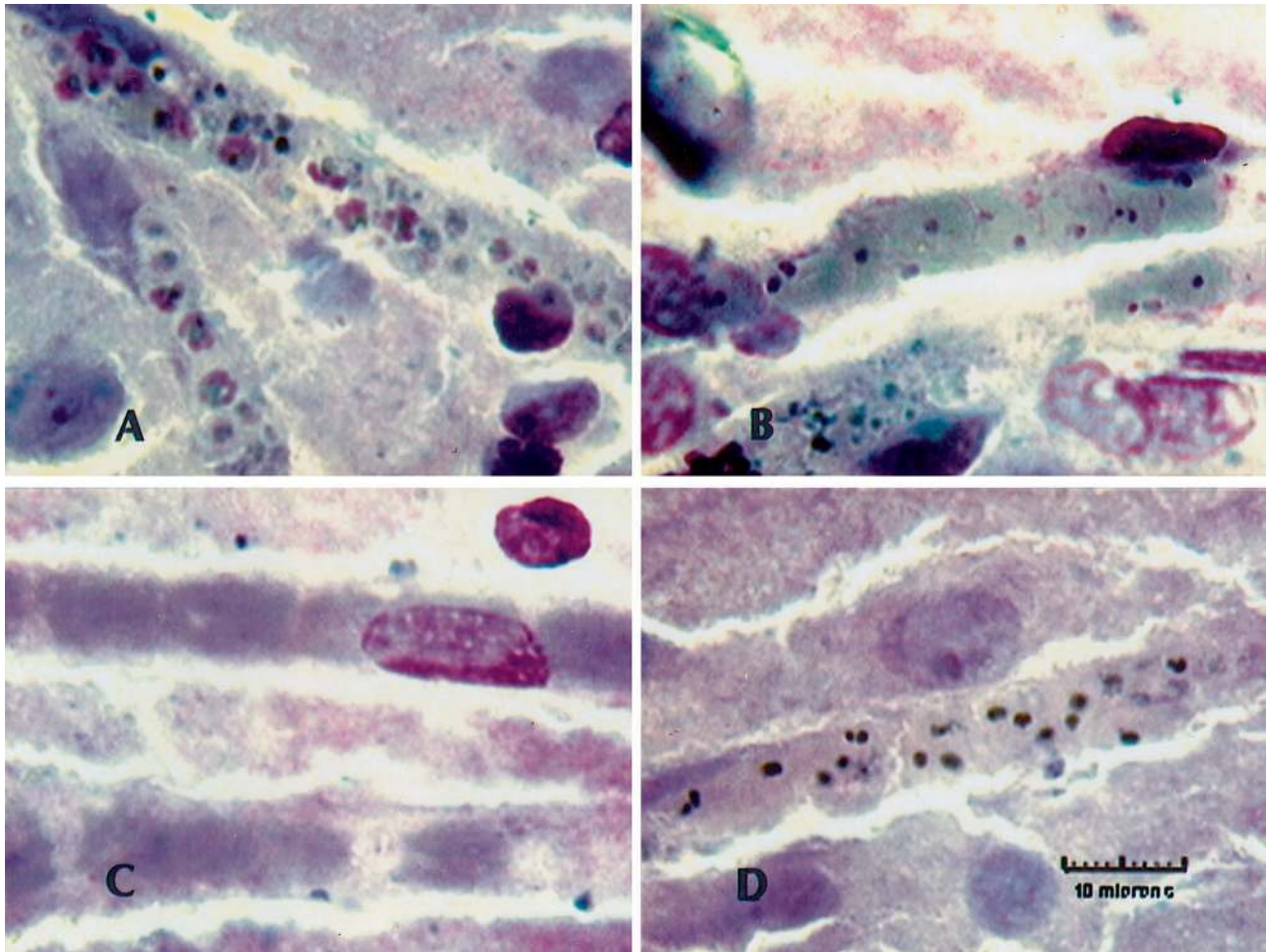


Figure 6. Four different areas from the same patient showing vessels with (A) late trophozoites and early schizonts (A) ring stages (B) uninfected red cells (C) no malaria parasites but residual intravascular pigment (D). The patient was treated with artemether and died 50 hours after admission. The peripheral parasite count at death was 400/ μ l.

Control Brains

The control brains showed a reproducible cerebrovascular endothelial cell immunophenotype similar to that seen in previous reports.²¹ Brain microvascular endothelium expressed class I HLA and CD31 constitutively and strongly. Focal scattered staining of most vessels for ICAM-1 and thrombospondin was also seen, the latter in a granular intracytoplasmic perinuclear pattern. P-selectin staining was also focal, cytoplasmic and granular, suggesting a resting storage pattern in Weibel Palade bodies. Very focal and weak CD36 and VCAM-1 staining was seen in some control cases, but many vessels did not express these antigens. E-selectin was uniformly negative in blood vessels, suggesting that this antigen is not expressed on normal brain endothelial cells, but variable expression was seen on some neuronal dendrites. Focal sparse chondroitin sulfate staining was seen on endothelial cells in two control cases, but stronger staining was seen on some perivascular macrophages. In general we did not observe CS positivity on control brain endothelial cells. These results are summarized in Table 2. Control brain endothelial immunophenotype was thus class I

HLA⁺⁺, CD31⁺⁺, class II HLA^{+/-}, ICAM-1^{+/-}, TSP^{+/-}, CD36⁻, E-selectin⁻, VCAM-1⁻, P-sel^{+/-}, CS⁻.

Malaria Brains

The staining patterns of putative host sequestration receptors on vessels in brains from fatal cases of malaria differed to that in the controls. The proportion of cases showing staining, and the intensity of staining was compared between control and malaria cases (Table 2, Figures 8 and 9). The intensity and distribution of ICAM-1 staining was increased, with nearly all vessels showing moderate to strong positive staining. Endothelial staining for class II HLA was increased and there was increased staining for VCAM-1 and E-selectin in a scattered pattern. Chondroitin sulfate staining was also increased, with a larger number of cases showing endothelial staining (Figure 8A) compared to the strong perivascular monocyte staining in controls (Figure 8B). Staining with thrombospondin appeared to decrease, perhaps reflecting release of this antigen from intracellular stores during endothelial activation. There was no change in the strong

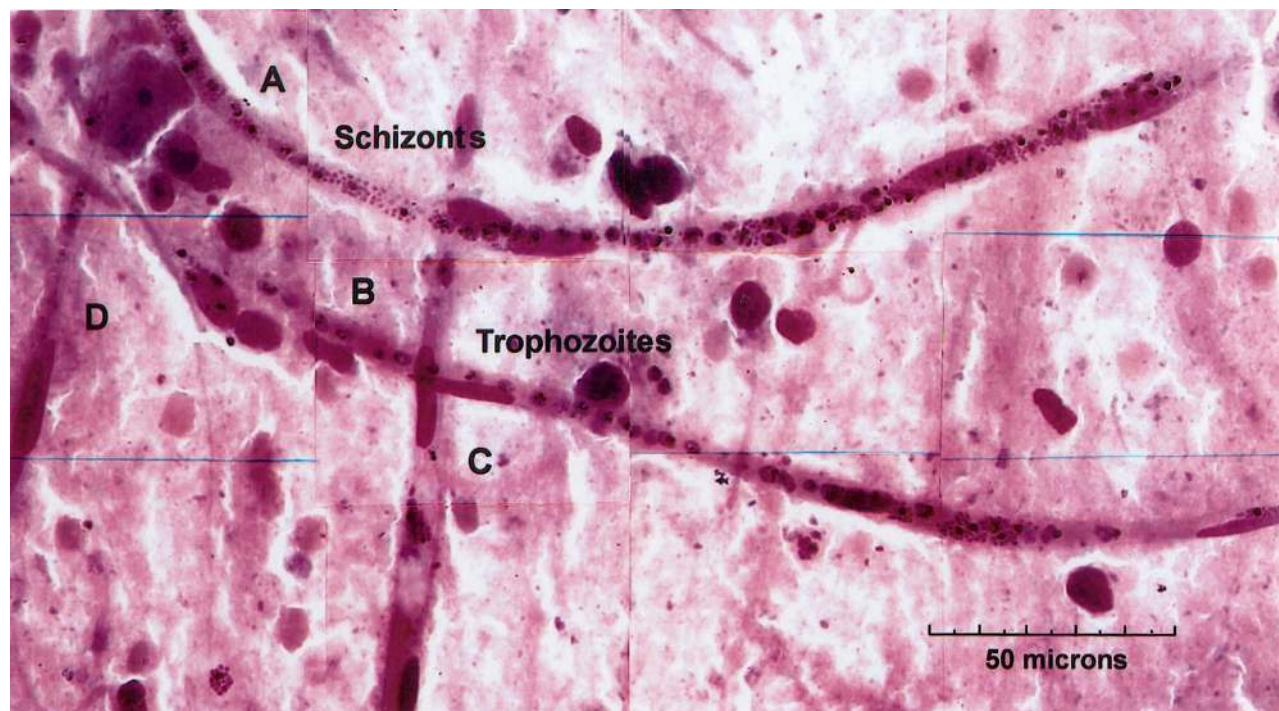


Figure 7. Composite photomicrograph (magnification, $\times 1000$) of a brain smear from a fatal case of falciparum malaria. Four vessels are seen (A–D). Nearly all the red cells in A and B are parasitized, whereas few parasites are seen in C and D. A is intensely parasitized containing alternating clusters of trophozoites and schizonts. Residual adherent pigment in the far right of the vessel indicates earlier schizont rupture. In B younger trophozoites are seen evenly spaced on the left and schizonts on the right. The patient received artemether but died 4 hours later after the parasitemia had doubled from 14.5% to 34.9%.

and constitutive expression of CD31 and class I HLA (Figure 8C), and no increase in the patchy, low levels of CD36 and P-selectin staining (Figure 8D). Thus endothelial cells in the brain of malaria cases show increased expression of ICAM-1, VCAM-1, E-selectin, CS, and class II HLA, and decreased TSP.

No specific changes in endothelial cell phenotype could be related to the presence of parasites in a particular vessel. CD31 staining did not appear to vary in terms of its distribution on the endothelial lumen. CD31 and ICAM-1 staining was widespread, and as such were quantitatively the only markers widely distributed enough to account for the bulk of parasitized erythrocyte sequestration. However, areas of focal staining for other potential receptors such as VCAM-1 and CS were also seen. Within vessels from the same patient, heterogeneity in parasite sequestration compared to the presence of re-

ceptor staining was seen. Thus whereas generalized endothelial activation was present in many vessels as judged by increased expression of ICAM-1 or VCAM-1, this did not equate with uniform parasite sequestration in all vessels (Figure 9, A–D). Accurate staging of parasite development was not possible in the immunohistochemical sections as parasite morphology was less well preserved, but with rough assessments (based on size, intraparasitic malaria pigment, and number of nuclei) the variation in endothelial staining along a vessel did not appear to be related to the stage of development of the locally sequestered parasites. There were no evident differences between the eleven cases of cerebral malaria and the two fatal malaria cases who did not have cerebral malaria. Both these cases also had variable increases in ICAM-1, VCAM-1, and E-selectin staining. Some vessels had patchy class II staining on some endothelial cells

Table 2. Comparison of Immunohistochemical Staining between Controls and Malaria Cases

Antigen	Staining pattern in control cases*	Staining pattern in malaria cases*	Changes
CD31/PECAM	100 (+)	100 (+ to ++)	No change
Class I HLA	100 (++)	100 (++)	No change
Class II HLA	50 (+/-)	100 (+)	Increased % cases and intensity
ICAM-1	90 (+/-)	100 (+ to ++)	Increased intensity
VCAM	50 (- to +/-)	69 (+/-)	Increased % cases and intensity
E-selectin	0 (-)	38 (- to +/-)	Increased % cases and intensity
CD36	40 (-)	38 (-)	No change
Thrombospondin	100 (+/-)	61 (+/-)	Decreased % cases
Chondroitin sulfate A	20 (-)	61 (+/-)	Increased % cases and intensity
P-selectin	60 (+/-)	61 (+/-)	No change

*Percentage of cases showing staining and average score.

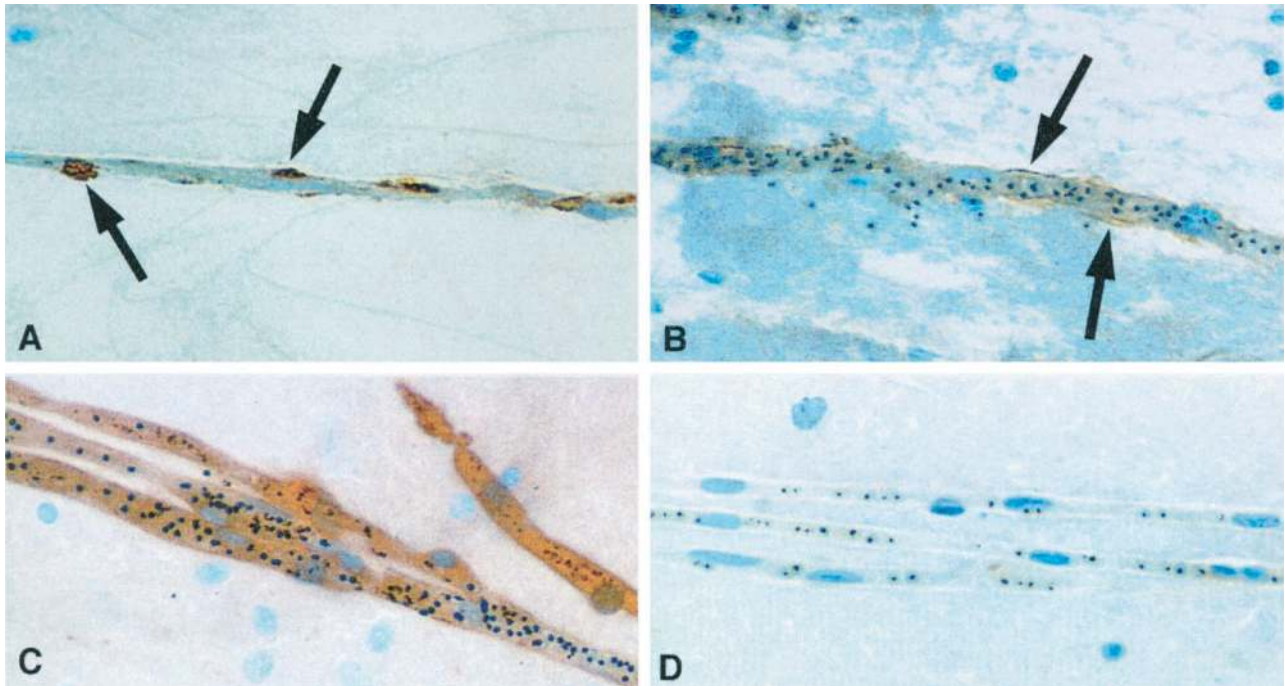


Figure 8. Immunohistochemical staining of cerebral vessels from brain smears. **A:** Chondroitin sulfate staining from control case; note the focal sparse CS staining on endothelial cells compared to stronger staining on peri-vascular macrophages (arrows). **B:** Chondroitin sulfate staining on a vessels from a cases of cerebral malaria. Increased endothelial staining is seen (arrows) compared to control staining. **C:** Strong constitutive endothelial class I HLA staining. **D:** Negative CD36 staining on vessels from a malaria case.

(Figure 9E), but in general if a vessel showed positive staining for a marker of endothelial activation, this was present throughout its length, rather than being related to the presence of PRBC in one segment or over one endothelial cell. There was little evidence for lymphocyte or platelet accumulation in vessels in any case. Scattered monocytes were seen in several malaria cases (CD36⁺, class I, and II HLA⁺ staining), and numerous class II HLA positive microglial cells were seen (Figure 9F).

Discussion

Shortly after the discovery of the malaria parasite by Laveran, malaria researchers in Rome noticed the discrepancy between the number and stages of parasite development in the peripheral blood and the brains of patients who died from cerebral malaria.²² Only the younger parasite stages of the potentially lethal *P. falciparum* were seen in peripheral blood smears, whereas the more mature parasites were abundant in the cerebral vessels. In contrast all stages of parasite development were represented in the peripheral blood of patients with the benign malarias. This process, known as sequestration, is not uniform. The intensity of sequestration differs between different organs,^{10,12,15} and also differs between the different parts of the brain.²³ As noted sequestration is not a feature of the other human malarias (*P. vivax*, *P. malariae*, *P. ovale*), in which all stages of parasite development may be seen in peripheral blood smears. In this series one patient died from vivax malaria, but there was no evidence of cerebral sequestration in this case. The present study provides formal mathematical proof of

sequestration in lethal falciparum malaria, and shows that sequestration of *P. falciparum* infected erythrocytes varies considerably even at the microvascular level.

It is possible that the preparation of brain smears leads to overestimation of sequestration, because capillaries without cytoadherent red cells may be missed on microscopic examination, or may be more likely to empty during the preparation of the brain smear, than those with sequestered parasites. However, this is unlikely to be a major confounder as capillaries and venules showing no evidence of cytoadherent parasitized erythrocytes were often seen, whereas others in the same section, several microns away, were intensely parasitized (Figures 5 and 6). Assessment of the stage of parasite development *in vivo* is also confounded by the effects of antimalarial drug treatment and compression within the confines of narrow vessels. However, *in vitro* and animal experiments indicated that these would not lead to large errors in stage assessment. The heterogeneity in the microvascular distribution of parasitized erythrocytes in fatal malaria emphasizes the importance of the vascular endothelial receptors for parasitized red cell cytoadherence in determining the extent and distribution of sequestration. Cytoadherence is mediated by the binding of parasite derived adhesins, expressed on the surface of the parasitized erythrocyte and also possibly modifications in the host cell membrane, to vascular endothelial cell receptors.^{1,24} The principal parasite adhesin is PfEMP1, a high molecular weight, antigenically variant product of the *var* gene superfamily.²⁵⁻²⁷ Multiple vascular receptors for cytoadherence have been identified *in vitro*. Quantitatively CD36 appears to be the most important receptor in

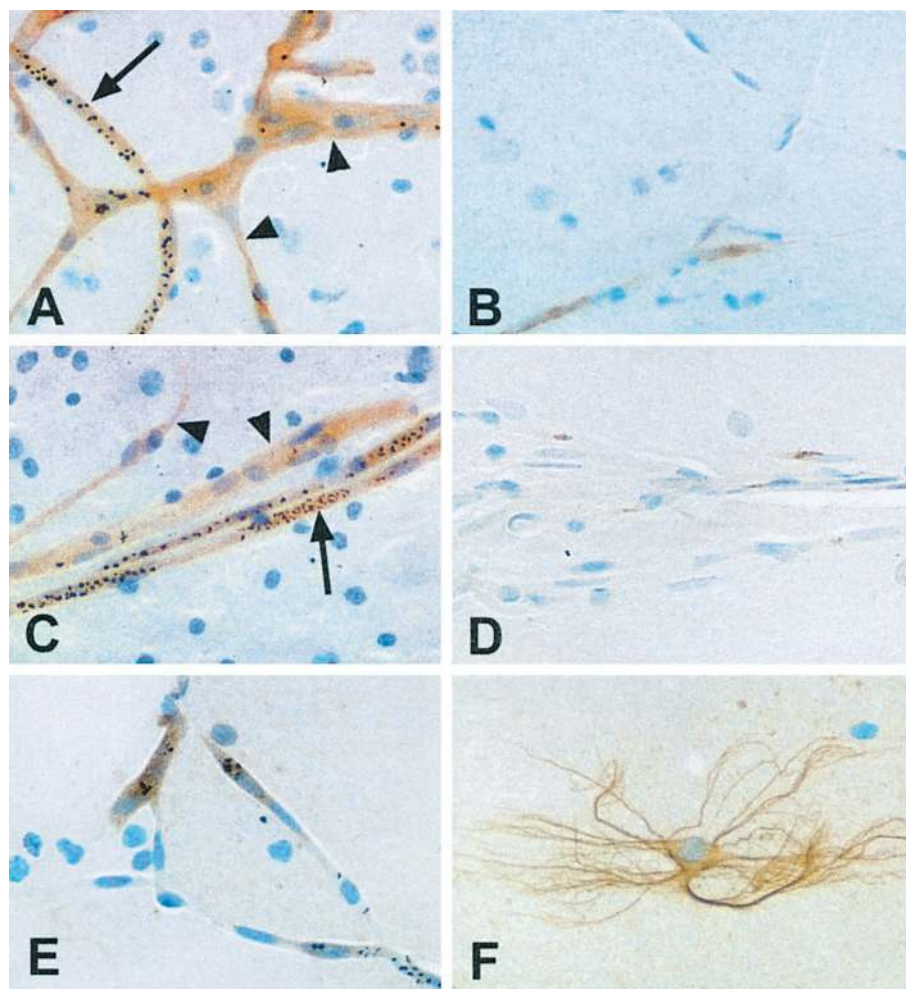


Figure 9. Immunohistochemical staining in malaria-infected brain smears. **A:** Endothelial activation in a malaria infected brain as judged by increased ICAM-1 expression. An ICAM-1 positive vessel with associated sequestration (**arrow**) is shown with neighboring ICAM-1 positive vessels and no sequestration (**arrowheads**). **B:** Control case showing patchy resting ICAM-1 expression on cerebral vessels. **C:** Increased VCAM-1 staining in a malaria infected cases, showing variation in sequestration between neighboring vessels both with (**arrow**) and without (**arrowheads**) sequestration. **D:** Very low levels of patchy endothelial VCAM-1 staining in a control case. **E:** Patchy class II HLA staining on some endothelial cells unrelated to the presence of PRBC in one segment or over one endothelial cell. **F:** A dendritic astroglial cell from a smear of a cerebral malaria case showing strong class II HLA staining.

the body, although this molecule is not generally expressed on the surface of cerebral vascular endothelium. ICAM-1 is probably the most important receptor for cytoadherence in the brain.²¹ Thrombospondin, E-selectin, VCAM-1, CD31/PECAM, and chondroitin sulfate A^{28,29} have also all been shown capable of mediating cytoadherence *in vitro*. Expression of some of these cytoadherence receptors is up-regulated by proinflammatory cytokines. This immunocytochemical study confirms our previous report that cerebral microvascular expression of ICAM-1, E-selectin, and VCAM-1 is increased in fatal malaria (Figure 9), and also shows for the first time that chondroitin sulphate and HLA class II expression is increased. CD-36 and P-selectin staining was very scanty. These receptors do not appear to mediate intracerebral sequestration (although CD36 is probably the major sequestration receptor outside the brain).

Changing expression of these ligands, and thus changing vascular endothelial cell receptivity for parasitized cell adhesins, is the most likely explanation for the uneven microvascular distribution of cerebral sequestration observed in this study. However, testing this hypothesis by relating the distribution of microvascular sequestration to different receptor types in autopsy specimens is problematic. Up-regulation can occur before or after se-

questration. Mature trophozoites or schizonts in a vessel have been sequestered for hours, or if arrested by anti-malarial treatment, for days before death. The increased expression of inducible receptors such as ICAM-1, VCAM-1, and E-selectin could result from systemic processes, such as the increased levels of proinflammatory cytokines, or from local changes related to microvascular obstruction and ischaemia. The finding that increased receptor expression tended to be present throughout a "sequestered" vessel, and not just at the site of parasitized red cell adherence, argues against the up-regulation of these receptors being solely a direct result of binding of PRBC to a particular cell. However, this does not preclude the possibility that binding could induce phenotypic changes in an individual cell, which would increase its adhesive potential. Also, there was no definite difference between patterns of staining in cerebral and non-cerebral malaria cases. These findings confirm that endothelial activation in the brain is not specific to cerebral malaria,³⁰ and would support the hypothesis that endothelial cell activation can occur independent of PRBC binding.

Absence of a putative endothelial receptor at a site of sequestration may not exclude its involvement in cytoadherence if that receptor is transiently up-regulated and

turned over rapidly. Because each case reflects a single time point during the disease process, which varies with each patient, differences in the temporal expression of particular receptors could be missed. However the co-expression of markers of endothelial activation such as E-selectin and VCAM-1, which have very different time courses of expression on endothelial cells *in vitro*, suggests that *in vivo* they are being subjected continuously to waves of activation on a more chronic time course, which does allow us to see both being expressed at the same time.

The parasites within the cytoadherent erythrocytes in each vessel or section of a vessel tended to be of similar age, whereas the distribution of parasite stages in the vessels overall was much broader, ie there was spatial clustering in parasite age. Presumably small blood vessels increase their receptivity to cytoadherence by increasing expression of these vascular ligands and select for those circulating parasitized erythrocytes newly capable of adhesion. All the red cells infected by more mature parasites would already be adherent (ie not circulating). Thus as the parasites mature and begin to express red cell surface adhesins, they are filtered out by those vessels newly capable of accepting them. Rolling is followed by static cytoadherence.³¹ Blood flow is not stopped by this process. There must be significant flow even through vessels partially occluded by cytoadherent parasitized erythrocytes to allow this filtration to continue, although the unparasitized erythrocytes presumably undergo considerable deformation in squeezing past the adherent and relatively nondeformable sequestered red cells.¹ This is the only explanation to account for the considerably higher proportion of parasitized red cells in the cerebral vessels compared to the peripheral blood. This study also suggests that once parasitized red cells adhere, they do not detach again and circulate freely. If they did recirculate this would lead to mixing of stages, and the parasites sequestered in each vessel should reflect the broad stage distribution of malaria parasites in the second 24 hours of their development.

Heterogeneity in microvascular sequestration in the brain may well explain the rarity of permanent neurological damage in cerebral malaria. It is likely that microvascular obstruction and consequent ischaemia provides a strong stimulus to vasodilation.³² Blood flow through those capillaries and venules which are not obstructed by parasitized cells would be expected to be maximal. Altered vessel wall shear stresses would also induce release of the potent vasodilator nitric oxide.³³ It has been suggested that nitric oxide may cause coma in cerebral malaria, although the source hypothesized has been cytokine up-regulated, inducible isoform of nitric oxide synthase (iNOS), rather than vascular wall endothelial isoform (eNOS), or the neuronal isoform (nNOS).^{2,3,34} Although there may be a background increase in vascular endothelial nitric oxide generation because of the systemic increase in proinflammatory cytokine release,³⁵ local factors are likely to predominate before terminal shock develops. Release of vasodilatory mediators would be expected to be greatest adjacent to cytoadherent red cells. Thus delivery of oxygen and metabolic substrates

in areas with partially occluded vessels may be sufficient to prevent permanent neuronal cell death. Even apparently blocked capillaries may still allow passage of uninfected red cells.

Although cerebral malaria was associated with intense sequestration within the brain, this was also seen in patients who did not lose consciousness. This may reflect the time between admission and death and does not disprove the concept that cerebral malaria is associated specifically with cerebral sequestration. Other patients in this series, particularly those who died after several days of antimalarial treatment, had few or no residual parasites in their cerebral vessels. This has been noted previously^{36,37} and has led some investigators to suggest that cerebral sequestration is not a necessary prerequisite for cerebral malaria.³ Nearly all such cases reported had received many days of treatment, and in this series residual pigment was evident within the cerebral microvasculature, even if no parasites were seen (Figure 10). Thus this relatively large study provides no support for the contention that cerebral malaria can occur in the absence of cerebral sequestration. Sequestration occurs in all patients with falciparum malaria and is a consistent pathological feature of severe malaria. If it was not then schizonts and mature trophozoites would be seen regularly in peripheral blood films. The absence of parasites from the brains of fatal cases indicates effective antimalarial treatment and clearance of infected erythrocytes. Such treatment cannot always reverse malaria related or secondary pathological processes in severely ill patients.

In this investigation, compared with the number of trophozoites recorded, we found less than the expected number of schizonts in the cerebral microvasculature. Previous *in vitro* and *in vivo* studies have suggested that the schizont or segmenter stage lasts for at least 10 hours.³⁸ Thus in a series of fatal cases, the number of schizonts and mature trophozoites sequestered would be expected to be approximately similar assuming a random distribution of parasite stages at the time of death. Schizonts are thought to remain sequestered until fully mature and then to burst leaving a cytoadherent red cell ghost. The number of trophozoites documented in this study was nearly four times greater than that of the schizonts. There are several possible explanations for this finding. Schizogony may be significantly shorter than estimated currently. Data from continuous *in vitro* culture suggest that this is very unlikely. It is possible that schizont rupture *in vivo* occurs in a proportion of cases much earlier than thought (ie before 18–24 merozoites have developed). Another explanation would be premature detachment of cytoadherent schizonts. The surface of schizonts are thought to contain less PfEMP1 molecules than mature trophozoites. If schizonts do detach, then they are not seen in peripheral blood films, and they do not appear to adhere again in the brain. It is possible they are cleared by the spleen, although semi-quantitative assessments of the parasitized cell population in the spleen indicates that all parasite stages are found, without an unusual predominance of schizonts.¹² Alternatively it is possible that the more metabolically active mature trophozoite stage is particularly virulent for the host, and patients may be

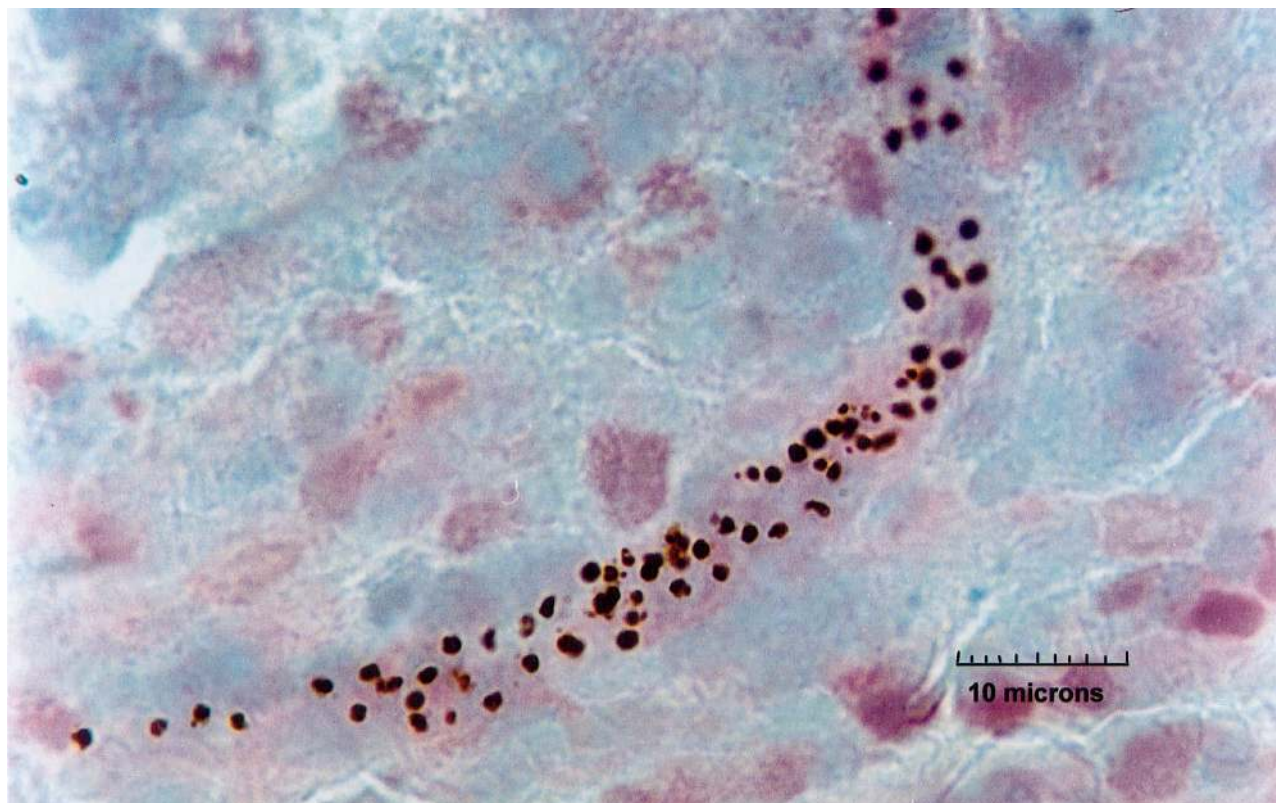


Figure 10. Brain vessel showing no malaria parasites but residual malaria pigment. The patient died after several days of quinine treatment. The peripheral parasite count at death was 80/ μ l.

more likely to die when maximum sequestration of this stage of parasite development occurs. Perhaps the most likely explanation for the apparent excess of trophozoites is their relative sensitivity to antimalarial drug treatment. The mature trophozoites stage is considerably more sensitive to antimalarial drugs than the schizont stage.^{39,40} Thus the parasiticidal effects of antimalarial treatment may prevent further development of the sequestered trophozoite stages. Indeed the artemisinin derivatives prevent development of the younger stages of parasite development but these are less likely to cytoadhere following this treatment,⁴⁰ and so this effect would not be reflected in the cerebral microvasculature.

Although schizonts were under represented in this study, tiny and small ring form parasites were over-represented in the cerebral vessels. These stages are not thought to cytoadhere. If it is assumed that there is free mixing within the systemic circulation of nonadherent red cells,⁴¹ the proportion of young ring forms in the cerebral vessels should be smaller than that in the peripheral blood, where the denominator number of unparasitized red cells is larger. The reverse was found. Indeed there were over 10 times more young rings in the cerebral vessels than would be expected from a free mixing model. Could this be an artifact of the stage assessment of drug exposed parasites in cerebral vessels? The staging of malaria parasites by light microscopy is not very precise because of the difficulties in obtaining very tightly synchronized cultures, different growth rates, smearing, and staining, and the simple differences from one para-

site with another. Compression of the parasitized erythrocytes within the cerebral capillaries could further confound interpretation. Although the red cell and parasite dimensions and shape were different in blood and brain smears, the amount of parasite cytoplasm, the number of nuclei, and, in particular, the presence of intraparasitic pigment were similar in simulation experiments in the rat. Antimalarial drug treatment also affected parasite morphology, and whereas quinine has little effect artemether significantly alters ring stage morphology and arrests development. However none of these confounders is likely to explain the presence of large numbers of small parasites without visible malaria pigment in the cerebral vessels. Malaria pigment, once formed, is insoluble and undeformable and is not resorbed.⁴² Intraparasitic pigment visible under light microscopy usually indicates that the parasite is more than 18 to 24 hours old.⁴¹ The absence of visible pigment is strong evidence that the parasite is in the first half of the asexual life cycle.

These data suggest that there is either sequestration or retention of the younger parasite forms in the cerebral microvasculature. Massive synchronous merogony would be an unlikely alternative explanation for this finding, and could not explain the grouping of small and large ring forms seen in some vessels. If schizogony occurred in a completely occluded vessel then a higher proportion of multiple infections would be anticipated, and some evidence of adjacent mature parasites or residual pigment should have been seen, which was usually not the case. It is possible that changes in surface charge or

membrane fluidity in ring stage infected red cells could alter their normal transit through the microcirculation and lead them to accumulate, preferentially in branch vessels which are normally closed to flow. Whichever the mechanism, red cells containing young rings, hitherto thought incapable of sequestration, are concentrated considerably in the cerebral vessels. Whether this is because of adhesion to vascular endothelium or to other parasitized cells or simply reflects their reduced deformability is not known, but it does suggest that peripheral parasite counts are an even greater underestimate of the parasite burden in severe falciparum malaria than previously thought.

Acknowledgments

We thank the Director and staff of the Center for Tropical Diseases, Cho Quan Hospital, Ho Chi Minh City and Paholpolpayuhasena Hospital, Kanchanaburi for their support for these studies. We are particularly grateful to doctors T.T.H. Chau, T.M.E. Davis, N.P.J. Day, S. Krishna, P.P. Loc, S. Pukrittayakamee, D.X.T. Sinh, W. Supanaranond, T.T.M. Trang, and D.J. Waller for help with clinical management.

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