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Expression of Merozoite Surface Protein Markers by *Plasmodium falciparum*-Infected Erythrocytes in Peripheral Blood and Tissues of Children with Fatal Malaria▽

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Sequestration of *Plasmodium falciparum*-infected erythrocytes is a pathological feature of fatal cerebral malaria. *P. falciparum* is genetically diverse among, and often within, patients. Preferential sequestration of certain genotypes might be important in pathogenesis. We compared circulating parasites with parasites sequestered in the brain, spleen, liver, and lung in the same Malawian children with fatal malaria, classifying serotypes using antibodies to merozoite surface proteins 1 and 2 and immunofluorescence in order to differentiate parasites and to quantify the proportions of each serotype. We found (i) similar distributions of various serotypes in different tissues and (ii) concordance between parasite serotypes in peripheral blood and parasite serotypes in tissues. No serotypes predominated in the brain in cerebral malaria, and parasites belonging to a single serotype did not cluster within individual vessels or within single tissues. These findings do not support the hypothesis that cerebral malaria is caused by cerebral sequestration of certain virulent types.

Malaria is a leading cause of morbidity and mortality in the developing world, with an estimated 1.5 to 2.7 million deaths per year (3). The burden of the disease is greatest in children less than 5 years old, and much of the mortality is attributable to cerebral malaria (CM). *Plasmodium falciparum* is the only human malaria species that causes fatal CM and the only human malaria species in which mature parasites sequester in deep microvasculature, strongly suggesting that sequestration plays a role in the pathogenesis of CM (31, 39, 40, 43, 50). Sequestration of parasitized red blood cells (pRBC) in tissues is a normal event in *P. falciparum* infection. Only young asexual ring stages are normally detected in blood by microscopy, whereas older stages (schizonts and trophozoites) are found in organs at autopsy.

P. falciparum is genetically diverse, and infections are mostly multiclonal, as widely demonstrated by PCR or serological typing using genetically polymorphic markers such as alleles encoding merozoite surface protein 1 (MSP-1) and MSP-2 (9, 11, 14, 26, 37, 47). Different genetic populations of pRBC, defined by *P. falciparum MSP-1* and *-2* types, may appear and disappear at intervals from peripheral blood independent of each other due to sequestration of maturing parasites of each clone on different days (4, 12, 16).

It is possible that genetically distinct pRBC could preferentially sequester in particular vascular beds and that a subset of *P. falciparum* genotypes is responsible for severe malaria, especially CM (22), but this hypothesis cannot be fully tested in humans in vivo. As part of an ongoing study of the clinicopathological correlates of fatal malaria, Montgomery et al. carried out a PCR analysis of *P. falciparum* genotypes at the *MSP-1* and *MSP-2* loci in blood and tissues of dying children with parasitemia; this analysis suggested that although multiple clones were present, they were distributed homogenously throughout the body (37). However, the PCR method employed did not provide relative quantification of the different genotypes, nor did it distinguish between circulating and sequestered parasites, as stages cannot be discriminated at the DNA level. To further elucidate whether specific parasite types sequester exclusively or predominantly in the brain, we investigated this question at the expressed protein level by performing an analysis that overcame some of the limitations of PCR. We used immunofluorescence typing with antibodies specific for different allelic types of MSP-1, MSP-2, and exported protein 1 (EXP-1) to identify *P. falciparum* serotypes (the protein corollary of genotyping [47]) in tissues. Compared to PCR, the immunofluorescence technique has the advantage that antibodies detect *P. falciparum* proteins expressed by trophozoites and schizonts, and thus it is particularly suited to identifying phenotypes of mature parasites sequestered in tissues. In

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TABLE 1. Antibody reagents for immunofluorescence antibody test typing

Antigen	Specificity	Antibody	Dilution	Isotype[a]	Domain or epitope	Reference(s)[b]
MSP-1_{19} block 17	K1/Well	111.4	500	IgG1/κ	EGF-1, "Q" dependent	24
	MAD20	6E2/53	100	IgG1/κ	EGF-2, "T-SR" dependent	44
	Conserved	12.8-2-1	500	IgG2b/κ	EGF-1, ^{1465}K-F^{1608}	32
MSP-1_{42} blocks 6 to 16	K1/Well	6.1-1-3	500	IgG1	^{1412}IETLY-TKFLS^{1491}	21, 34
	K1/Well	13.1-2	500	IgG1	^{1412}IETLY-TKFLS^{1491}	21, 34
	K1/Well	17.1-3	1,000	IgG1	^{1412}IETLY-TKFLS^{1491}	21, 34
	MAD20	9.2-6-2	2,000	IgG1	^{1078}NSLNNPHN-RVSGSSGS^{1251}	33
MSP-$1_{42/36}$ blocks 6 to 16	Conserved	9.8-4-4-2	500	IgG1	Conformational	33
	Conserved	12.4-3-4	500	IgG1	Conformational	33
	K1/Well	7.3-7	500	IgG2a	Conformational	34
	MAD20	9.7-1	500	IgG1	Conformational	34
	MAD20	10.3-2	2,000	IgG1	Conformational	34
MSP-1_{80} block 4b	K1/Well	10-2B	1,000	IgG2a/κ	^{331}TLLDKNKKIEE^{342}	9, 10, 25
	MAD20	12.1-5-4	2,000	IgG1	^{334}PLPENKKKEVEG^{345}	9, 10, 33
MSP-1_{80} block 3	K1/Well	13.2-3	1,000	IgG1	"^{124}R-E^{284}" dependent	9, 10, 32, 34
	MAD20	9.5-1-5-1	1,000	IgG2b/κ	"^{127}Q-Q^{287}" dependent	9, 10, 34
MSP-1_{80} block 2	K1	12.2-1-1	1,000	IgG1	3D7 repeats $(SAQ)_n$	34
		123D3	500	IgG2b/κ	Palo Alto repeats $(SGT)_n$	29
		CE2.1	60	IgG1/κ	Palo Alto repeats $(SGT)_{nn}$	28
		Polyclonal[c]	1,000		Palo Alto and 3D7 block 2	6
	RO33	31.1-5[d]	2,000	IgG1/κ	^{65}PADAVSTQ-SDAKSYADL^{116}	McBride
		31.2-2-6[d]	2,000	IgG1/κ	^{65}PADAVSTQ-SDAKSYADL^{116}	McBride
		Polyclonal[c]	800		RO33 block 2	6
	MAD20	Polyclonal[c]	200		MAD20 and Well block 2	6
MSP-2 block 4	A (IC1)	12.3-2-2	500	IgG1	Group specific dimorphic	34
		12.5-1-2	500	IgG1	Group specific dimorphic	34
MSP-2 block 3		12.7-1-2-4	500	IgG1	Group specific dimorphic	34
MSP-2 block 3, R1		113.1	500	IgG3	Polymorphic repeat GSAGS	48
		113.2	100	IgM/κ,λ	Polymorphic repeat GSAGS	48
		4-4F	200	IgM/κ	Polymorphic repeat GSAGS/GGSA	8
		8-5D	200	IgM/κ	Polymorphic repeat GSAGS/GGSA	8
		13.4	1,000	IgG1	Polymorphic repeat T9/94 GGSA	McBride
		Polyclonal anti-$TTn_{5/7}$[c]	300		Polymorphic repeat GASGRAGA	McBride
		Polyclonal anti-$7G8_{5/7}$[c]	400		Polymorphic repeat GGSGSA	McBride
		Polyclonal anti-$T9/96_{5/7}$[c]	300		Polymorphic repeat GAVAGSGA	McBride
MSP-2 block 3, R1	B (FC27)	8G10/48	1,000	IgG2b/κ	Group specific dimorphic STNS	44
		Polyclonal anti-$K1_{5/3}$[c]	300		Group specific dimorphic	McBride
		Polyclonal anti-$T9/105_{12/6}$[c]	200		Group specific dimorphic	McBride
MSP-2 block 3, R2		8F6/49	50	IgG3/2b	Polymorphic repeat DTPTATE	44
		Polyclonal anti-$K1_{13/3}$[c]	100		Polymorphic repeat DTPTATE	McBride
	Conserved	Polyclonal anti-$K1_{17/14}$[c]	200		Conserved	McBride
EXP-1	K1	5.1-4	500	IgG1	Dimorphic amino acid 136	46
Knobs	Conserved	18.2-3-3	100	IgG2b		McBride
	Conserved	18.2-4	100	IgG2b		McBride
	Conserved	9.21-4-2	100	IgM		McBride

[a] Ig, immunoglobulin.
[b] McBride, J. M. McBride, unpublished data.
[c] Sera raised against recombinant MSP-1 and MSP-2 fragments in mice.
[d] MAbs raised against recombinant MSP-1

addition, in mixed infections, the relative proportions of each serotype in the blood and tissues can be determined by direct visualization of double-stained mixed parasite populations using fluorescence microscopy.

MATERIALS AND METHODS

Patient samples. Postmortem samples were collected from 15 children less than 12 years old who died at a pediatric research ward between 1996 and 1998. These patients had (i) CM (with a Blantyre coma score of <3 [36], asexual

parasitemia, and no other obvious cause, after correction for hypoglycemia and recovery from convulsion] ($n = 7$); (ii) CM and severe malarial anemia (hemoglobin level of <5 g/dl or hematocrit value of <15%) ($n = 3$); or (iii) coma or severe illness other than malaria (two had incidental parasitemia) ($n = 5$). On admission, we determined hematocrit and parasitemia levels. Thick and thin blood films were stained with Field's stain, and at least 200 leukocytes and 500 erythrocytes, respectively, were counted to record numbers and stages of parasites. Informed consent was obtained from parents or guardians, and ethical approval for the study was obtained from the investigators' institutions.

Blood parasites. For 10 of the 15 children, 5 ml of venous blood was obtained on admission. The blood was centrifuged to remove the buffy coat and plasma, and the erythrocytes were washed in sterile phosphate-buffered saline (PBS). Seven children had asexual parasites as determined by blood smears, and short-term in vitro cultures of six samples that had levels of parasitemia of >5% were set up. pRBC from three patients with fatal cases were successfully matured to express MSPs and knobs (11, 49), and three cultures failed to grow due to previous antimalarial treatment. Parasitized erythrocytes were harvested from in vitro cultures and washed twice in PBS. Cells were resuspended to 3 to 5% hematocrit in PBS, and 20- to 25-μl aliquots were placed onto wells of 12-well multispot slides (Hendley-Essex, United Kingdom). The slides were dried, packed, and stored at −20°C in self-sealing plastic bags containing silica gel as a desiccant. Residual pRBC were stored at −70°C for analysis of parasite DNA.

Immunofluorescence antibody typing (IFAT). Type-specific antibodies identify distinct allelic forms of *P. falciparum* MSP-1, MSP-2, and EXP-1 and thus can detect the presence of multiple-serotype mixed infections in individuals (9). The serological reagents used included monoclonal antibodies (MAbs) and polyvalent mouse antibodies specific for *P. falciparum* MSP-1 and -2 and EXP-1 (Table 1). Three known MSP-1 block 2 types, represented by the RO33, MAD20, and K1 isolates, were identified by specific MAbs (11, 28) and/or polyclonal antibodies (6). Other MAbs identified MSP-1 polymorphisms in blocks 3 and 4, in dimorphic regions (blocks 6 to 16) of the MAD20 type or Well/K1 type, and conserved epitopes (block 17) (9, 10). Polymorphisms between and also within the two major serogroups of MSP-2, serogroups A and B, were similarly identified by MAbs (17) or antibodies against recombinant MSP-2. The typing method described previously was used (9, 11). Briefly, slides with pRBC were fixed in acetone, and 25-μl portions of working dilutions of typing antibodies were placed in separate wells and incubated for 30 min at room temperature in a wet box. After the antibodies were removed, the wells were washed three times with PBS, and the slides were dried on a warm plate, 15 μl of fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin (1/80) was placed in each well, incubated, and washed as described above. Parasite DNA was stained with 4′,6′-diamino-2-phenylindole (DAPI) (1:100,000) for 5 min, and the blood films were counterstained with 0.1% Evans blue. After 5 min, the slides were rinsed with PBS and mounted under coverslips with Citifluor (City University, London United Kingdom) or 50% glycerol in PBS. Reactions were read by using a magnification of ×315 or ×630 and incident light at wavelengths of 450 to 490 nm for FITC fluorescence (green) or 390 to 440 nm for DAPI (blue). Serological marker epitopes are referred to below by the same code numbers as the MAbs that were used to identify them. For each isolate and MAb, the percentage of parasites exhibiting antibody-specific positive fluorescence was recorded.

Multiple-clone infections were analyzed by double-labeled (two-color) IFAT using combinations of two MAbs for different isotypes against different epitopes, as described previously (9, 10, 15) (Table 1). Briefly, FITC- and rhodamine isothiocyanate (RITC)-conjugated anti-mouse immunoglobulins (1:50) specific for the isotypes of the typing MAbs were incubated together in the second stage. The slides were not counterstained with Evans blue. Reactions were read by using incident light at wavelengths of 515 to 560 nm for RITC fluorescence (red). The proportions of schizonts showing (i) only green (and blue) fluorescence, (ii) only red (and blue) fluorescence, (iii) red and green (and blue) fluorescence, and (iv) neither red nor green (only blue) fluorescence were recorded for each pair of MAbs tested. Combined results, obtained with a series of different pairs of MAbs, resolved the number of distinct parasite clones within each isolate and the phenotypes. At least 200 schizonts per test were scored.

PCR genotyping. *P. falciparum* DNA extracted from 20 to 100 μl erythrocytes by the quick boiling method (18) was used in a hot-start nested PCR to analyze the multiplicity of infection (MOI). Block 2 of *MSP-1* was amplified with primers O1 and O2 (outer reaction) (41) and a set of block 2 type-specific primers (inner reaction) (6). Dimorphic regions of *MSP-1* and of *MSP-2* were typed by the dimorphic form-specific PCR method (42).

Autopsy specimens. Postmortem specimens were obtained from 14 autopsies and a supraorbital needle sample (patient SO). Parasitemia was recorded less than 4 h before death for 13 of 15 patients, and the times between admission to the hospital and death and between death and autopsy were noted for all cases. Tissue biopsies from the cerebral hemisphere, cerebellum, lung, heart, liver, kidney, and spleen were processed in four different ways (1). Homogenates were prepared by grinding a piece of tissue (2 by 2 by 1 cm) with a pestle and mortar in PBS, washing the material twice in PBS, and placing 20 to 25 μl of the suspension onto 12-well multispot slides (2). Smears were prepared using small pieces of tissue crushed between two slides which were then pulled apart (3). Imprints were prepared by pressing a slide against a section of the organ. All slides were dried and stored at −20°C for IFAT (4). Pieces of tissue that were 7 by 3 by 3 mm were covered in Tissue-Tek optimal cutting temperature compound (Ted Pella Inc., Redding, CA) and flash frozen. Eight-micrometer frozen sections of cryopreserved tissue on slides were kindly provided by Georges Grau, Geneva, Switzerland. To assess the degree and stage of parasite sequestration, brain smears were fixed in methanol, stained with Giemsa or Field's stain, and examined with an oil immersion lens (magnification, ×100). Parasites sequestered in tissues were typed by immunofluorescence analysis, as described above (9, 15).

RESULTS

Detection of sequestered parasites. Using light microscopy (Fig. 1A) and IFAT, we found mature parasites sequestered in the brain, spleen, liver, and lung but not in the heart or kidney in nine CM patients who died. No sequestered parasites were detected in five non-CM patients who died. The presence of sequestered parasites in CM patients who died was confirmed by the colocalization of fluorescent staining of *P. falciparum* DNA and of surface proteins with antibodies. Homogenates, smears, and cryosections (Fig. 1) of brain tissues were suitable for IFAT typing. Although the morphology of the tissue was disrupted by homogenization, most capillaries containing pRBC remained intact, and smears preserved individual parasite morphology. Cryosections had the advantage that the histological structure was maintained. Estimation of the proportions of serotypes in multiple infections was easier with smears and cryosections than with homogenates, as capillaries did not clump together. Organ imprints were more appropriate than homogenates for the lung, spleen, and liver (Fig. 2).

Immunofluorescence serotyping of sequestered parasites. Mature parasites sequestered in tissues from 10 CM patients were typed using a panel of antibodies specific for MSP-1, MSP-2, and EXP-1 (Table 2). All these parasites also reacted positively with knob-specific MAbs (not shown). The antigen phenotypes of parasite clones of each isolate and their relative proportions were determined by combining the results obtained with different pairs of antibodies. Antibodies that did not react positively in any of the samples indicated antigenic types that were not expressed in the parasites examined (MAD20 type MSP-1_{17}, K1 type MSP-$1_{6\text{-}16}$, and particular polymorphic repeat epitopes of MSP-2) and are not included in Table 2.

In addition to schizonts, in most cases trophozoites were also present in the tissues, as detected by Giemsa staining and IFAT (Fig. 1 and 2). Thus, sequestered pRBC contained mostly somewhat asynchronous parasite broods, and parasites at different stages were present at each tissue site. In such cases, trophozoites were detected by DNA staining but were negative for most typing antibodies, except those recognizing EXP-1 (antibody 5.1), knob antigens, and block 17 of MSP-1 (antibody 111.4), which are expressed earlier in the blood asexual cycle and are normally present on early trophozoites. In two patients, the sequestered parasites consisted of synchronous trophozoites that were negative for the MSP-1 (except antibody 111.4) and MSP-2 antibodies. Typing of these seques-

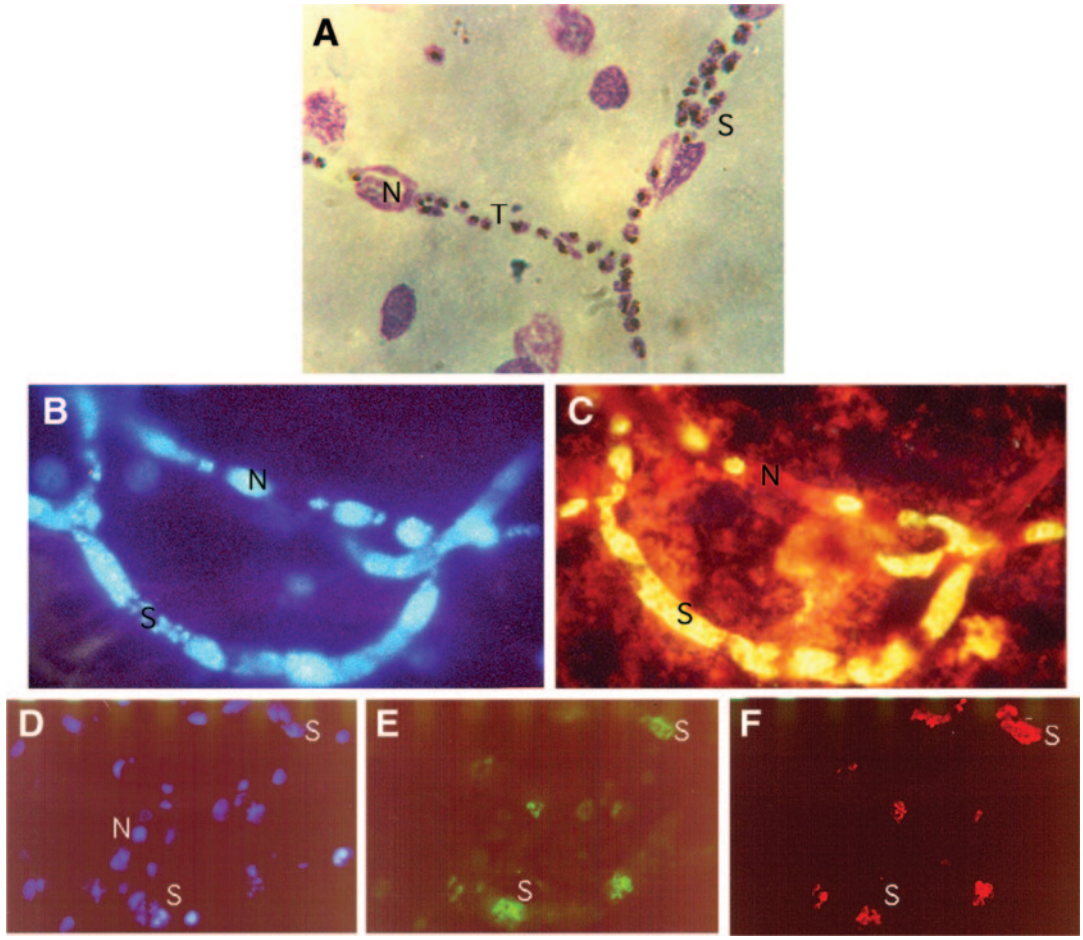

FIG. 1. Detection of parasites in brains from children who died from CM. (A) Thin smear prepared from brain tissue (patient 13), methanol fixed and Giemsa stained, examined with a light microscope, showing capillaries with sequestered segmenter schizonts (S), trophozoites (T), and malaria pigment. The pink oval bodies are the nuclei (N) of the capillary endothelial cells. Magnification, ×630. (B) Brain homogenate (patient 6) showing capillaries with sequestered *P. falciparum* schizonts stained with DAPI. The blue dots correspond to the DNA of the multinucleated schizonts (S), and the blue oval bodies correspond to endothelial cell nuclei (N). Magnification, ×315. (C) Same sample as the sample in panel B, stained with FITC. Sequestered *P. falciparum* schizonts (S) were recognized by MAb 18.2, specific for a malaria knob antigen, at exactly the same positions at which blue fluorescent dots are present in panel B. Note that the lower capillary appears to be full of parasites, while the upper capillary is almost empty. N, nuclei. (D to F) Cross sections of cryopreserved brain (patient 6). (D) Capillaries containing fluorescent schizonts stained with DAPI. Blue round bodies correspond to brain cell nuclei (N) and sequestered schizonts (S). Magnification, ×315. (E) FITC staining of the sample in panel D, stained with MAb 113.2 (immunoglobulin G3) specific for MSP-2 serogroup A. Only schizonts (S) are recognized by this MAb. (F) RITC staining of the sample in panel D, stained with MAb 5.1 (immunoglobulin G1) specific for EXP-1. Both schizonts (S) and trophozoites are recognized by this MAb. For details concerning the methods and antibody reagents used see Materials and Methods and Table 1.

tered parasites was thus restricted to EXP-1, which distinguished between parasites from patients 16 (positive) and 21 (negative).

MOI in tissues. Parasites found in tissues often belonged to more than one *P. falciparum* serotype (Table 2 and Fig. 3). Using double-labeled IFAT typing, we determined the proportion of each serotype in multiple infections in the cerebral hemisphere and cerebellum (Table 2) and, whenever possible, in the spleen. The spleen, liver, and lung generally contained fewer parasites than the brain, and thus it was possible to determine only the presence or absence of each antigenic type, without quantification or phenotype resolution. For the eight CM cases in which tissue parasites could be serotyped, there was one single infection, three cases with at least two serotypes, and four cases with at least three serotypes. The MOI was 2.37, which is similar to the MOI determined by PCR for the blood of 93 CM patients who did not die admitted during the study period (MOI, 2.25) (15).

Parasite serotypes in tissues and blood. Partial within-patient comparisons of tissue-sequestered serotypes in the spleen, liver, lung, and brain could be performed for seven autopsies (Table 2). Owing to the limited availability of spleen, liver, and lung specimens and the lower intensity of sequestration, a smaller panel of antibodies to dimorphic epitopes was selected for typing parasites detected in these tissues. In patient 27, the same serotypes were detected in the spleen, liver, and lung as in the brain, and they were in the same proportions in the spleen and the brain. For the other six patients only a partial comparison could be made, but we did not detect any serotype in the spleen, liver, or lung that was not also present

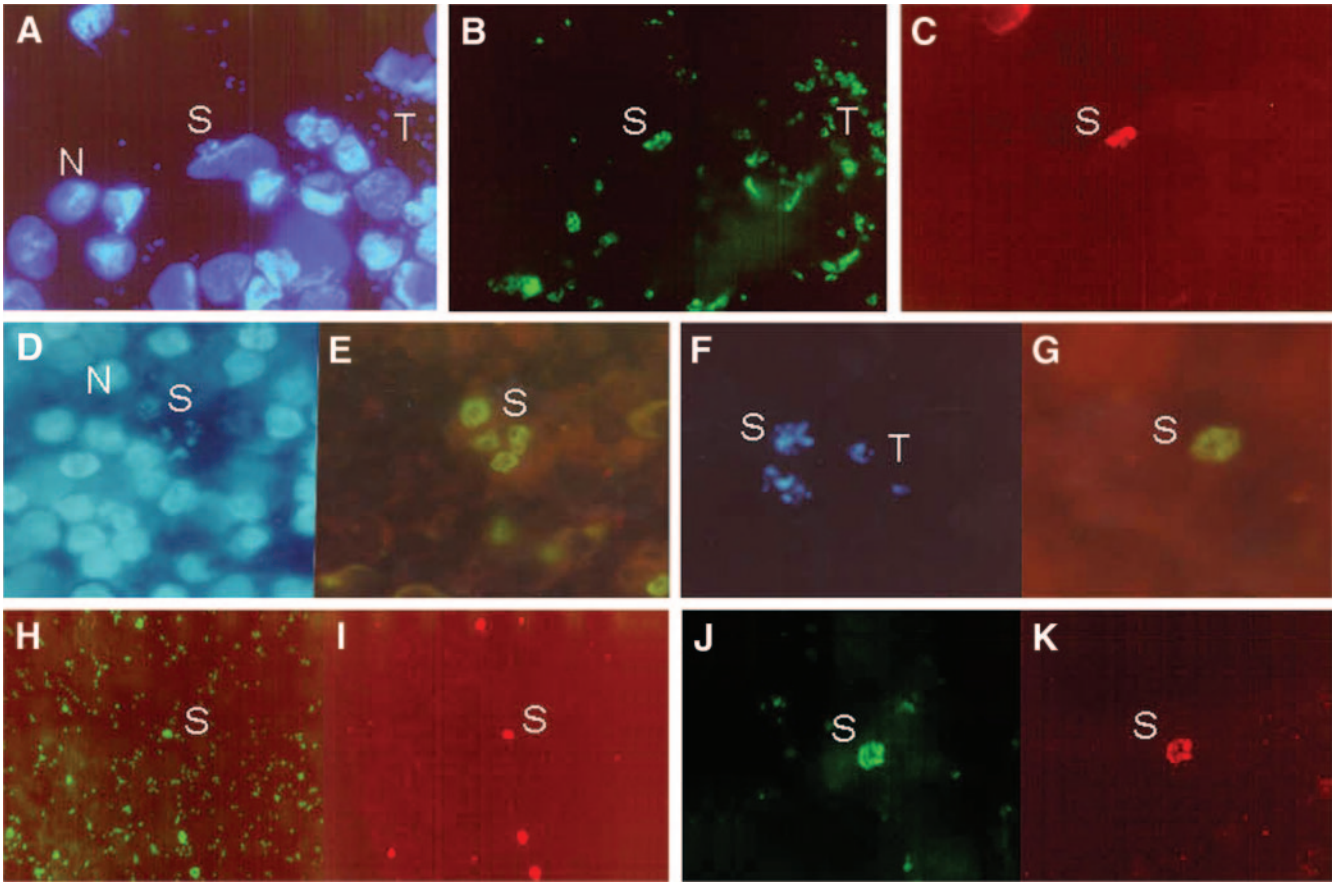

FIG. 2. Detection of parasites in organs of children who died from CM. (A, B, and C) Lung imprints. (A) Parasites detected in a lung imprint (patient 27) stained with DAPI. Trophozoites (T) and one schizont (S) are surrounded by lung cells. N, nuclei. Magnification, ×630. (B) Sample in panel A stained with FITC-conjugated MAb 111.4 (immunoglobulin G1) specific for an epitope in block 17 of MSP-1 expressed in both trophozoites (T) and schizonts (S). (C) Sample in panel A stained with RITC-conjugated MAb 123D3 (immunoglobulin G2b) specific for the K1 type (Palo Alto variant) of block 2 of MSP-1. Only one schizont (S) is recognized by this MAb. (D and E) Splenic imprints. (D) Fluorescent schizonts (patient 6) stained with DAPI. The blue dots in the center (S) correspond to schizont nuclei. The blue round bodies (N) correspond to the nuclei of spleen cells, Magnification, ×630. (E) Sample in panel D stained with FITC-conjugated MAb 9.8 (immunoglobulin G) specific for conserved epitopes of MSP-1. S, schizont. (F and G) Hepatic imprints. (F) Four parasites, two schizonts (S) and two trophozoites (T), were detected with DAPI (patient 13). Magnification, ×630. (G) Sample in panel F stained with FITC-conjugated MAb 31.1 (immunoglobulin G) specific for epitopes of the RO33 block 2 type of MSP-1. Only schizonts were detected by MSP-1-specific MAbs. Trophozoites were negative as determined by FITC fluorescence. More than one *P. falciparum* clone was identified in sequestered parasites from patient 13 (Table 2), and the clones were distinguished by different MSP-1 block 2 types. The FITC-fluorescent schizont (S) belonged to the RO33 block 2 type, whereas the negative unstained schizont belonged to the K1 block 2 type. (H) Fluorescent schizonts (S) detected in another spleen imprint (patient 27) stained with FITC-conjugated MAb 111.4 (immunoglobulin G1) specific for epitopes in block 17 of MSP-1. Both trophozoites and schizonts are recognized by this MAb. Magnification, ×315. (I) Spleen sample in panel H stained with RITC-conjugated MAb 123D3 (immunoglobulin G2b) specific for the K1 type (Palo Alto variant) of block 2 of MSP-1. Only schizonts (S) are recognized by this MAb. (J) Fluorescent schizonts (S) detected in another liver imprint (patient 27) stained with FITC-conjugated MAb 111.4 (immunoglobulin G1) specific for epitopes in block 17 of MSP-1. Magnification, ×630. (K) Liver sample in panel J stained with RITC-conjugated MAb 123D3 (immunoglobulin G2b) specific for the K1 type (Palo Alto variant) of block 2 of MSP-1. S, schizont.

in the brain of the same patient. In summary, in each case, the parasites detected in the spleen, liver, or lung were the same MSP-1/2 and/or EXP-1 types as the parasites in the other organs and in the brain; therefore, we concluded that within each patient there was the same qualitative distribution of sequestered *P. falciparum* types.

Between-patient comparisons of tissue-sequestered pRBC revealed some similarities in the serotypes detected (Table 2). Thus, all parasite clones belonged to one of two main dimorphic serotypes of MSP-1 (MAD20), none belonged to the alternative main type (K1), and most expressed the same epitopes in blocks 2 (predominantly the K1 type), 3, and 4 (Table 2). For MSP-2, most CM patients were infected with serotype A parasites (IC1-like); serotype B (FC27-like) was found in only one patient in tissues. Finally, epitope 5.1 of EXP-1 was positive in nearly all cases; the only exception was patient 27. Overall, these epitope prevalences in CM patients who died reflected the prevalences also found predominantly in the blood of children with nonfatal cerebral or uncomplicated malaria in Blantyre (15).

Quantitative comparisons of the serotype proportions in the brain and blood could be made for patients 6 (two serotypes)

TABLE 2. Typing of tissue and blood parasites in fatal cerebral malaria cases

Patient	Infection	%	MSP-1 antigen										MSP-2 antigen blocks 3 and 4					
			Block 17, type K1 (antibody 111.4)[a]	Blocks 6 to 16, type MAD20 (antibodies 9.2, 9.7, and 10.3)	Block 4		Block 3		Block 2				Type A				Type B (antibodies 8G10/48, $K1_{5/3}$, and $T9/105_{12/6}$)	EXP-1 antigen (dimorphic, type K1, antibody 5.1)
									Type K1		Type RO33 (antibodies 31.1 and 31.2 and polyclonal sera)	Type MAD20 (polyclonal sera)						
					Type K1 (antibody 10-2B)	Type MAD20 (antibody 12.1)	Type K1 (antibody 13.2)	Type MAD20 (antibody 9.5)	Antibodies 3D7 and 12.2	Antibodies PA, 123D3, and CE2.1			Antibodies 12.3, and 12.5, 12.7	Antibodies 113.1 and 113.2	Antibodies 4-4F and 8-5D	Polyclonal TTn		
6	Brain	95	+++[b]	+++	+++	−	+++	−	+/−	+++	−	−	+++	+++	+++	−	−	+++
	Brain mixed	5	−	+++	+++	−		−	−	−	++	−	+++	−	−	−	−	+++
	Spleen	ND	+++	+++	+++	−	ND	ND	ND	ND	ND	ND	+++	ND	ND	ND	−	+++
	Liver	ND	+	+	+	−	ND	ND	ND	ND	ND	ND	+	ND	ND	ND	−	+
	Lung	ND	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+
	Blood	95	+++	+++	+++	−	+++	−	+/−	+++	−	−	+++	+++	+++	−	−	+++
	Blood mixed	5	−	+++	+++	−		−	−	−	++	−	+++	−	−	−	−	+++
11	Brain	90	+++	+++	+++	−	+++	−	+++	−	−	−	+++	−	−	−	−	+++
	Brain mixed	10		+++	−	+	−	++	−	−	++	−	+++	−	−	−	−	
	Spleen	ND	+	+	+	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+
	Liver	ND	+/−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+/−
	Lung	ND	+/−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+/−
13	Brain	65	−	+++	+++	−	−	−	−	+++	−	−	+++	−	+++	−	−	++
	Brain mixed	30	+++	+++	−	+++	−	+++	−	−	+++	−	+++	++	+++	−	−	−
	Brain mixed	5	−	+++	+++	−	−	+++	+/−	+++	−	−	+++	−	−	−	−	
	Spleen	ND	++	++	++	++	ND	ND	ND	ND	ND	ND	++	ND	ND	ND	−	++
	Liver	ND	+	+	+	+	ND	ND	ND	ND	ND	ND	+	ND	ND	ND	−	+
	Lung	ND	+/−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+/−
	Blood	75	−	+++	+++	-	−	−	−	+++	−	−	+++	−	+++	−	−	+++
	Blood mixed	20	+++	+++	−	+++	−	+++	−	−	+++	−	+++	++	+++	−	−	−
	Blood mixed	5	−	+++	+++	−	−	+++	+/−	+++	−	−	+++	−	−	−	−	
15	Brain	50	−	+++	+++	−	−	−	−	+++	−	−	+++	−	−	−	−	+++
	Brain mixed	30	++	+++	−	+++	−	+++	−	−	+++	−	+++	−	−	−	−	
	Brain mixed	20	−	+++	+++	−	++	−	+++	−	−	−	+++	−	−	−	−	
	Spleen	ND	++	++	++	++	ND	ND	ND	ND	ND	ND	++	ND	ND	ND	−	++
	Liver	ND	+/−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+/−
	Lung	ND	+/−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+/−
16	Brain	NA	+++	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	+++
	Spleen	NA	++	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	ND
	Liver	NA	++	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	ND
	Blood	75	−	+++	+++	−	+++	−	−	+++	−	−	+++	−	−	+++	−	+++
	Blood mixed	20	+++	+++	+++	−	−	+++	−	−	+++	−	−	−	−	−	+++	
	Blood mixed	5	−	+++	−	−	+++	−	−	+++	−	−	−	−	−		+++	
21	Brain	NA	+++	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	−
	Spleen	NA	++	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	ND
	Liver	NA	++	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	ND
23	Brain	60	−	+++	+++	−	+++	−	−	+++	−	−	+++	−	−	−	−	−
	Brain mixed	40	++	+++	+++	−	+++	−	+++	+++	−	−	+++	−	+/−	−	−	+++
26	Brain	100	+++	+++	+++	−	−	++	−	−	+++	−	++	−	−	ND	−	+++

27	Brain	60	+++	+++	–	–	–	–	–	–	+++	–	–	ND	–	–	–	–
	Brain mixed	30	+++	+++	–	++	–	–	+++	–	+++	++	++	ND	–	–	–	–
	Brain mixed	10	–	+++	–		–	++	–	–	+++	++	++	ND	–	–	–	–
	Spleen	60	+++	+++	–	–	–	–	–	–	+++	–	–	ND	–	–	–	–
	Spleen mixed	30	+++	+++	–	++	–	–	+++	–	+++	++	++	ND	–	–	–	–
	Spleen mixed	10	–	+++	–	–	–	++	–	–	+++	++	++	ND	–	–	–	–
	Liver	ND	+	+	–	+	–	–	+	–	+	+	+	ND	–	–	–	–
	Lung	ND	+	+	–	+	–	–	+	–	+	+	+	ND	–	–	–	–
SO	Brain	40	ND	+++	ND	ND	ND	–	–	+++	–	ND	ND	ND	ND	+++	ND	ND
	Brain mixed	30	ND	+++	ND	ND	ND	–	–	–	+++	ND	ND	ND	–	–	ND	ND
	Brain mixed	30	ND	+++	ND	ND	ND	++	–	–	+++	ND	ND	ND	–	–	–	ND

[a] See Table 1 for specificities of monoclonal antibodies or polyclonal sera against recombinant proteins.
[b] +++, very intense reaction with antibody against the antigen marker; ++, intense reaction; +, weak reaction; +/−, very weak reaction; −, not reactive; ND, antibody reactivity not tested owing to insufficient quantity of good-quality tissue sample.
[c] NA, not available for testing. In addition, data were not obtained for blood samples from patients 11, 15, 21, 23, 26, 27, and SO and for lung samples from patients 16 and 21.

and 13 (three serotypes) (Fig. 3). Table 2 shows the results of IFAT typing of blood isolates that matured into schizonts in vitro in the same patient for which tissue typing was performed. In these two autopsies, the same serotypes were detected with very similar proportions in both the blood and tissues. In patient 16, blood parasites could be cultured and serotyped (three serotypes), but it was not possible to compare these parasites to parasites in the brain because sequestered stages were too young (trophozoites) to be fully typed by MSP-1/2 antibodies.

For the rest of the cases, the blood samples were too small for *P. falciparum* culture or the parasites failed to mature (e.g., owing to prior antimalarial treatment), and thus we determined circulating genotypes by PCR analysis. Genotyping revealed that all patients who died had *P. falciparum* parasites in the peripheral blood on admission, and many were infected with multiple genotypes. A more detailed account of the PCR approach was provided in a previous study (37), and here this method was used as a supplement to protein typing to validate the comparisons in cases where IFAT with blood was not available. Thus, in patients 6, 13, and 16, PCR genotyping of blood was also performed, and the genotypes found corresponded to the serotypes shown in Table 2. For patient 16, PCR proved to be more sensitive than IFAT, as IFAT with blood quantified two serotypes (K1 and RO33) whereas PCR detected three genotypes (K1, RO33, and MAD20). For three patients, serotypes of sequestered parasites could be compared only with PCR-determined genotypes of circulating parasites. For patients 11 and SO we found the same multiplicity of infection in blood and tissues, and the genotypes of MSP-1 (polymorphic block 2 and dimorphic blocks 6 to 16) and MSP-2 (dimorphic) in blood matched the corresponding serotypes in tissues. In patient 15, the peripheral parasitemia was low, but the brain and spleen were full of schizonts. For this patient, IFAT detected three MSP-1 block 2 serotypes in the brain (K1, K1, and RO33) (Table 2), whereas genotyping of blood detected four *MSP-1* block 2 bands (K1, K1, RO33, and MAD20), even with the low parasitemia; a faint MAD20 PCR band in the blood was not identified as expressed protein in tissue, probably owing to the lower sensitivity of the IFAT method for detecting minority clones. In conclusion, in all cases examined, comparisons of serotypes and genotypes in tissues and blood showed that the compositions of MSP-1 and -2 types in tissues and peripheral blood were very similar and that the multiplicities of infection were consistent for parasites circulating in the blood and parasites sequestered in tissues.

DISCUSSION

Studies of the characteristics of *P. falciparum* sequestered in human tissues face many obstacles. Clinical definitions of CM (51) must be precise. Deaths are rare, and autopsies may be culturally unacceptable or technically impossible, or consent may not be granted. If comparisons to circulating parasites are planned, manyfold more samples must be prepared from peripheral blood to ensure that the samples corresponding to autopsy cases are captured, but obtaining suitable samples from critically ill children is challenging. Despite these obstacles, studies such as ours are critical to increasing our understanding of the pathogenesis of CM. In comparison, the recent

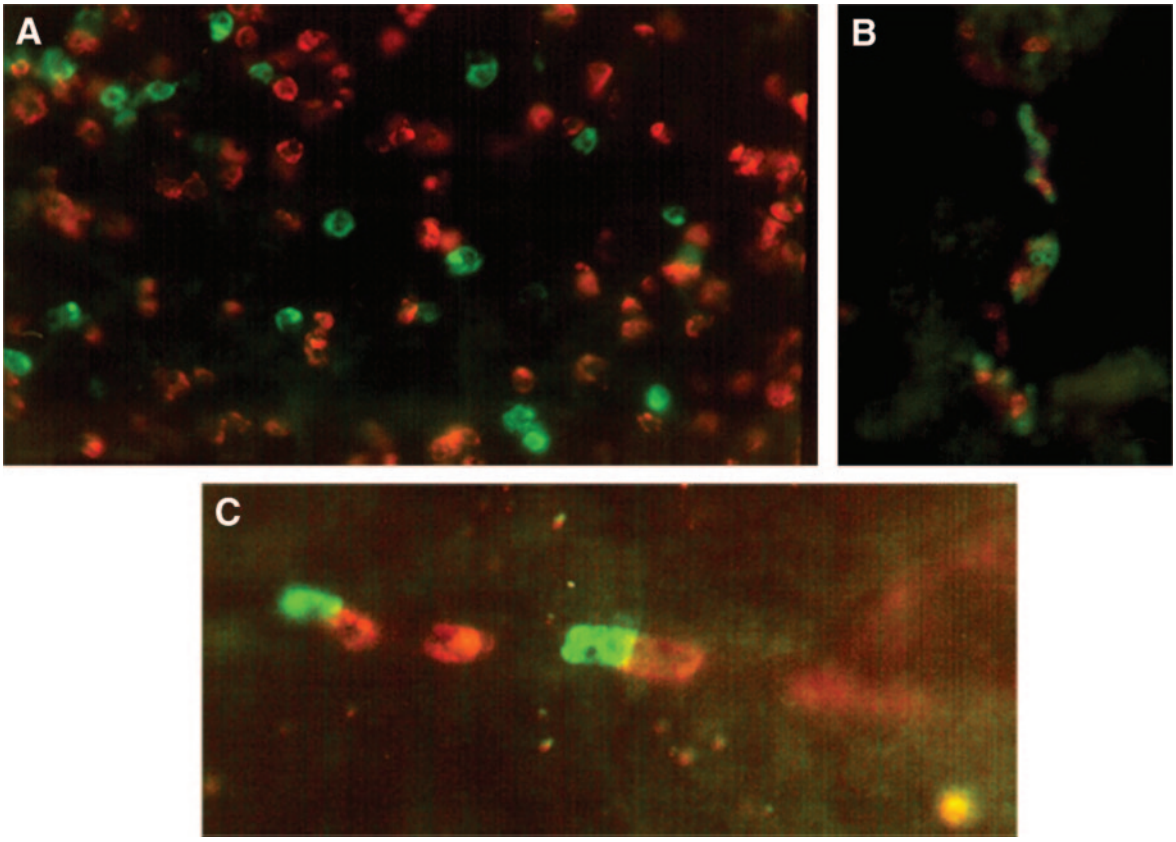

FIG. 3. Detection of multiple-clone infections in blood and brain by double-labeled IFAT: MSP-1 serotypes of *P. falciparum* in peripheral blood and brain tissue of patient 13. (A and B) *P. falciparum* schizont-infected erythrocytes cultured from the peripheral blood (A) and homogenate from the frontal lobe of the brain (B) of the same child. A majority of schizonts (red) reacted with MSP-1 block 4-specific MAb 10-2B (immunoglobulin G2a) plus RITC-conjugated anti-immunoglobulin G2a. A minority of schizonts (green) were positive with MAb 12.1 (immunoglobulin G1) plus FITC-conjugated anti-immunoglobulin G1. The detectable serotypes or relative proportions of sequestered and circulating parasites did not differ. Magnification, ×630. (C) Thin smear prepared from a supraorbital needle sample from patient SO, showing a mixed infection of two MSP-2 serotypes in a brain capillary. Some schizonts (red) reacted with MSP-2 group B-specific MAb 8G10/48 (immunoglobulin G2b) plus RITC-conjugated anti-immunoglobulin G2b, whereas others (green) reacted with MSP-2 group A-specific MAb 12.3 (immunoglobulin G1) plus FITC-conjugated anti-immunoglobulin G1. Magnification, ×630.

major advances in understanding placental malaria (2, 19, 20, 38) have been facilitated by the ready availability of human tissue samples.

Sequestration of pRBC in the brain may contribute to the pathogenesis of CM. The intensity of parasite sequestration in tissues has been correlated with the occurrence of CM (31, 37) and with the clinical coma score (43). If CM is due to virulent genotypes of parasites that cause pathogenesis by sequestering in crucial sites, we would expect to find some genotypes exclusively or predominantly in the brain rather than also in other tissues. This hypothesis was investigated within the context of the same autopsy-based study by Montgomery et al. (37), who found that specific genetic populations of pRBC, defined by *P. falciparum MSP-1/2* genotypes, were not associated with preferential sequestration in the brain. In the present study we used a different approach to study this question, determining parasite phenotypes and their quantitative distributions rather than genotypes. Thus, using tissue specimens from 10 autopsies and serological in situ typing of parasites for the well-characterized antigen markers MSP-1 and -2, we asked (i) whether parasite clones (referred to as serotypes here) sequestered in tissues could be identified and, in multiple infections, distinguished by this method and (ii) whether there was differential within-patient distribution of serotypes among the organs or between organs and the peripheral blood.

We first showed that immunostaining with MSP-1/2 and EXP-1 type-specific antibodies, as well as non-type-specific antibodies (knobs), could be used to visualize and distinguish serotypes of parasites sequestered in organs. Four different methods for preparing tissues for this study were evaluated, and specific procedures were recommended for each organ. Using fluorescence microscopy, we could determine the topographical locations of different parasite serotypes within tissues and even within vessels, as shown in Fig. 1 to 3. We then examined whether there was differential sequestration of particular serotypes in the brain compared to other organs. Although we and other workers (35, 39, 45) have observed a lack of uniformity in the distribution and load of sequestered parasites in the capillaries, here we showed by using double-labeled IFAT that specific genetically determined serotypes did not segregate in different organs. In the patients for whom it was possible to qualitatively compare parasites from different

organs, sequestered parasites had the same MSP-1/2 and EXP-1 serotypes in all tissues examined. In individuals infected with more than one genotype, one would expect a relationship between markers, limited in time and space to the particular event; therefore, it was important to perform quantitative comparisons for a patient using serotypic markers. In our assessment, when the proportions of the serotypes in a three-clone infection were determined and compared for the brain and spleen (patient 27), the frequencies of each clone were similar in the two organs. Moreover, within individual vessels mixed serotypes were found (Fig. 3C). Thus, in accordance with the findings of Montgomery et al. (37), we obtained no evidence for a subset of parasites predominating in the most affected tissue (i.e., the brain in CM) or for clustering of any one serotype within any vessel or tissue.

We next investigated whether there were differences in the distribution of *P. falciparum* serotypes between the peripheral blood and organs within the same patient. In the peripheral blood, the results of IFAT serotyping or PCR genotyping (when IFAT was not possible) were consistent with the results of phenotypic analyses of parasites in tissues. One important advantage of IFAT over PCR typing is that IFAT detects intact, mature parasites (sequestered), whereas PCR can amplify DNA from immature parasite stages that may be circulating through the tissue capillaries (probably not sequestered) or even DNA from dead parasites. Therefore, by examining the late-stage-specific proteins we obtained a more accurate picture of the composition of the types in tissues and in blood. Overall, our results indicate that the circulating parasite populations have the same mixture of parasite clones as the parasite populations sequestered in the brain and in other tissues. In asymptomatic children sampled frequently, parasite genotypes detectable in the peripheral blood show periodicity consistent with synchronous sequestration of individual genotypes, as defined by the MSP-1/2 markers used in this study (4, 16). Our data, by contrast, revealed similar numbers and proportions of serotypes in the peripheral blood and tissues and do not suggest that there is differential sequestration of individual serotypes. It may be that in more severe infections the synchronous infection pattern described here is lost.

Our data do not support the hypothesis that fatal CM is due to one or a few particularly virulent parasite genotypes that preferentially sequester in brain capillaries. First, multiple *P. falciparum* infections were common among the CM patients who died, and the multiplicity of infection did not differ significantly from the number of circulating clones that we have found in patients in Blantyre with nonfatal CM (15). Second, the MSP serotypes found in the CM patients who died were within the range of serotypes prevailing in Blantyre. Other studies using variant surface antigens to type circulating parasites also showed that severe malaria (5) and CM (27) in young children were associated with parasite types that were prevalent in the community and commonly recognized.

The evidence for variability in virulence among clones of *Plasmodium* is only indirect and comes mainly from classical malaria therapy studies with neurosyphilitic patients. More recently, some evidence has been obtained with *Plasmodium chabaudi* rodent models (30) and for *P. falciparum* in relation to in vitro growth rates that differed for different parasite isolates from patients with severe malaria and patients with uncomplicated malaria in Southeast Asia (7) but not in Africa (13) or in relation to the capacity of pRBC to adhere to multiple receptors (23). In our study we obtained no evidence which suggested that MSP-1/2 polymorphisms are implicated in CM pathogenesis or that these loci contribute to the site of sequestration of pRBC. This would not be expected, as MSPs function in erythrocyte invasion but are not exposed at the surface of pRBC or functionally involved in cytoadherence. Because of the genetic structure of *P. falciparum* populations (9, 11, 41), between-patient comparisons are likely to be revealing only if functional antigenic markers linked to the pathogenic process are used. *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (1) is thought to be differentially expressed at the surface of pRBC and to result in different receptor-ligand interactions and tissue-specific adhesion patterns. It is possible that clonally identical pRBC may sequester in different tissues because they express different PfEMP1 variants. The importance of PfEMP1 in determining the number of pRBC sequestering in individual organs is not known, and expression studies to address this question would require development of appropriate antibody and DNA probes beyond the scope of this project.

Despite the challenges faced by this kind of study and the technical limitations for performing thorough quantitative assessments, our data indicate that within a patient, all genotypes are found in all sites to the same degree. In conclusion, the similar distributions of parasite serotypes analyzed here using antisera to MSP markers in blood and tissues of children with fatal malaria do not support the hypothesis that CM is due to a few particularly pathogenic genotypes that preferentially sequester in the brain.

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