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Cryoglobulins, Circulating Immune Complexes, and Complement Activation in Cerebral Malaria

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A total of 32 patients with *Plasmodium falciparum* malaria were studied. Of these, 23 had benign infections, and 9 had typical cerebral malaria. Cryoglobulins, circulating immune complexes detected by a C1q-binding assay, and hypocomplementemia were found in eight of nine patients with cerebral malaria. Raised levels of complement component 3 breakdown products (C3d) were found in the seven patients tested. Peak levels of circulating immune complexes and C3d were associated with thrombocytopenia. In contrast, in patients with benign *Plasmodium falciparum* malaria, cryoglobulins and circulating immune complexes were found only in 3 of 23 patients. Similarly, hypocomplementemia was detected only in 5 of 23 patients. These observations suggest that the intensity of the immune response and of the associated complement activation may be important factors in the pathogenesis of cerebral malaria.

Soluble malarial antigens, as well as antibodies to various plasmodial constituents, have been demonstrated in the serum of patients infected with either *Plasmodium falciparum* (18, 28) or *Plasmodium malariae* (14). Similar findings have been made in monkeys infected by *Plasmodium brasilianum* (14) and in mice infected with *Plasmodium berghei* (15). As would be predicted from these observations, it has been established that malarial antigens could be present in the circulation, either free or bound to antibodies, giving rise to circulating immune complexes (CIC) (14), which potentially can be deposited in various vascular territories (2, 4, 5, 7-9, 13-16, 27). This sequence is suspected strongly in human cases of immune complex-type glomerulonephritis after *P. malariae* infection (16) as well as in the production of immune complex deposits in the choroid plexus and glomeruli of mice infected with *P. berghei* (15). The potential role of circulating immune complexes in other clinical manifestations of plasmodium infection has received little attention. In this study, we present some evidence suggesting that the presence of circulating immune complexes, cryoglobulinemia, and complement consumption in *P. falciparum* malaria are associated with cerebral malaria and very rarely with the uncomplicated infection.

MATERIALS AND METHODS

Patients. A total of 32 patients with *P. falciparum* infection presented themselves at Hôpital Claude Bernard in Paris. In all, the diagnosis was established by the presence in the blood of *P. falciparum*-infected erythrocytes and anti-*P. falciparum* antibodies. Of these patients, 23 (14 black, 9 caucasian) with benign disease were admitted for acute fever and never developed any clinical complications. Nine patients had typical cerebral malaria. The main clinical findings are summarized in Table 1. All had been recently in endemic areas (eight in Western Africa and one in the Comoro Islands and Tunisia) without taking any prophylactic treatment. A previous trip to an endemic area was recorded in seven of nine patients, and two had already had malarial infections. All patients were treated with intravenous quinine therapy for several days, followed by chloroquine given per os. Apart from one patient (no. 5) who died within 72 h, all patients recovered.

Cryoglobulins. Cryoglobulins were isolated from 20 ml of blood drawn in a warm tube and processed as previously described (1). The protein concentration of the cryoprecipitate was assessed by the method of Folin. The immunoglobulin content was studied by immunodiffusion, using monospecific antibodies to human γ, α, μ, λ, and κ chains of immunoglobulins (Centre Départemental de Transfusion Sanguine, Bois-Guillaume, France). A total of 500 μg of three cryoglobulins was dissociated by ultracentrifugation on a 10 to 40% sucrose gradient in 0.1 M acetate buffer (pH 4.5) in a Beckman SW.50 rotor at 175,000 $\times$ *g* for 15 h at 15°C. Fractions (0.2 ml) were collected and analyzed for protein concentrations, immunoglobulin content, and cryoprecipitability as described above, anti-*P. falciparum* antibodies, rheumatoid factor, and C1q-binding and C3-splitting activities.

Detection of CIC. The detection of CIC was performed by the radiolabeled C1q-binding assay by the method of Zubler et al. (32).

TABLE 1. *Main clinical features in nine patients with cerebral malaria*

Patient no. and sex	Age	Parasited erythrocytes (%)	Neurological symptoms	Renal failure	Mechanical ventilation	Hemorrhage	DIC[b]	Thrombocytopenia
1, ♂	29	12	Stupor, delirium	+	+	+	+	+
2, ♂	41	45	Light coma, meningeal syndrome	+, PD[a]	−	−	+	+
3, ♂	24	13	Deep coma	+, PD	+	+	−	+
4, ♂	53	20	Stupor	+	−	+	−	+
5, ♀	30	2, 5	Deep coma	−	+	+	−	+
6, ♂	26	0, 6	Stupor	+	−	−	+	+
7, ♂	39	1	Light coma	−	+	−	−	+
8, ♂	28	25	Stupor	+	−	+	+	+
9, ♂	32	0, 3	Stupor and extrapyramidal syndrome	+	−	−	−	+

[a] PD, peritoneal dialysis.

[b] DIC, disseminated intravascular coagulation.

Rheumatoid factor. Rheumatoid factor was detected, using human immunoglobulin G (IgG)-coated latex particles (Institut Pasteur, Paris.).

Anti-*P. falciparum* antibodies. Anti-*P. falciparum* antibodies were detected and titrated by indirect immunofluorescence on thin blood smears from patients with heavy *P. falciparum* parasitemia, using fluorescein-labeled monospecific anti-human IgG or IgM antibodies (Hyland Laboratories, Inc., Plaisir, France).

Complement component studies. For complement component studies (17), 50% hemolytic complement (CH50) was measured by hemolytic titration, using the technique of Mayer. C1q, complement component 4 (C4) and 3 (C3), factor B, properdin, and complement component 5 (C5) levels were measured by radial immunodiffusion, using commercially available antisera (Hoechst-Behring, Paris, and Atlantic Antibodies, Portland, Maine). Results were expressed as a percentage of a pool of normal human sera. In addition, factors B and D also were determined by hemolytic titration in agarose plates.

Complement activation. Complement activation was detected by measurement of C3d levels using the method described by Perrin et al. (22). The C3d of 0.02 M ethylenediaminetetraacetate plasma was separated from C3 by 15% polyethylene glycol precipitation. The C3d level was assessed in the supernatants by rocket immunoelectrophoresis, using an anti-C3d serum (Netherland Red Cross, Amsterdam). Results were expressed in micrograms per ml by reference to a standard preparation of pure C3d prepared in our laboratory by the method of Lachmann (17) and Perrin et al. (22). C3-splitting activity was detected by crossed immunoelectrophoresis (17).

RESULTS

Antibodies and parasitemia. All 32 patients had IgG anti-*P. falciparum* antibodies with titers ranging from 1:64 to 1:4,096. The mean level was higher in patients with cerebral malaria (1:660) than in patients with uncomplicated malaria (1:534), but this difference was not statistically significant. Heavy parasitemia was not a common feature in cerebral malaria (Table 1).

Cryoglobulinemia and circulating immune complexes. All nine patients with cerebral malaria had IgG-IgM cryoglobulinemia, ranging in concentration from 1 to 41 mg/100 ml (Fig. 1), and eight of nine had a raised C1q-binding activity, suggesting the presence in their serum of CIC (Fig. 2). There was a good correlation between the levels of CIC and the cryoglobulin concentrations, and both reached a peak 11 to 16 days after onset of symptoms. In seven patients, shortly after quinine therapy was initiated, there was a marked increase in cryoglobulin and CIC levels, associated in four instances with an increase in the severity of the coma. The biological properties of three cryoglobulins from patients no. 2, 4, and 5 were studied before and after acid dissociation on a sucrose gradient. All three cryoglobulins contained IgM κ and λ; two contained IgG κ and λ, and one contained IgG λ. Three showed C1q-binding activity and in vitro C3-splitting activity, using either normal human serum (3:3) or C2-deficient serum (2:3). The dissociated fractions of these three cryoglobulins were not cryoprecipitable and did not have any C1q-binding or C3-splitting activity. Rheumatoid factor was detected in the IgM peak. In patient no. 2, IgM and IgG anti-*P. falciparum* antibodies were detected. IgM antibodies were concentrated five times, and IgG antibodies were concentrated six times over the serum levels. In patient no. 5, only IgG anti-*P. falciparum* antibodies were detected, and they were concentrated two times. In patient no. 4, anti-*P. falciparum* antibodies were not detected.

In contrast with the observations made in patients with cerebral malaria, cryoglobulin and

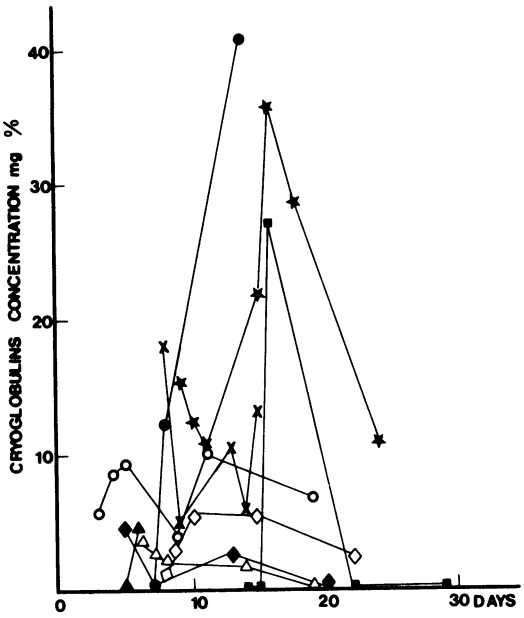

FIG. 1. *Amount of cryoglobulins (expressed on the ordinate as milligrams per 100 ml of serum) determined on sera obtained 2 to 36 days after onset of symptoms. Each patient is represented by a separate symbol: ◇, 1; ◆, 2; △, 3; ○, 4; ▲, 5; ■, 6; □, 7; X, 8; ●, 9. All patients, except one, had abnormally high C1q-binding activities.*

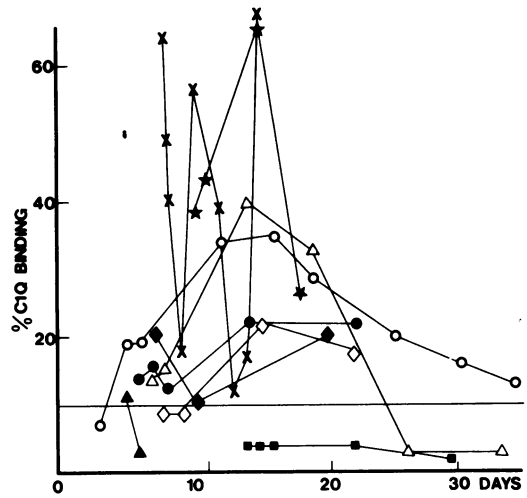

FIG. 2. *C1q-binding activity (expressed on the ordinate as a percentage of C1q precipitated) measured on sera obtained 2 to 36 days after onset of symptoms. Each patient is represented by a separate symbol as shown in the legend to Fig. 1.*

CIC were rare in patients with uncomplicated *P. falciparum* infection. Cryoglobulins were found only in 4 of 23 cases (with titers ranging from 0.8 to 18 mg/100 ml), and CIC were found in 3 of 23 cases.

Complement studies. Results of the complement studies are shown in Table 2 and Fig. 3. Marked hypocomplementemia was observed frequently in patients with cerebral malaria. Eight patients had low levels of CH50, and seven had low levels of C4 or C3 or both. C1q and properdin were less frequently decreased, whereas C5 and factor B were within normal limits. Hemolytic titrations of factors B and D also gave results within the normal range. All seven patients tested had increased levels of C3d, indicating in vivo complement activation. Complement abnormalities were present as early as day 3, when CIC and cryoglobulins were present only at low levels and lasted as long as 16 days, reaching a maximum around day 9 or 10. Maximum complement activation occurred with maximum levels of CIC and cryoglobulins. However, although complement components were usually normal by day 16, raised C3d levels lasted as long as CIC and cryoglobulins were detected. Finally, thrombocytopenia occurred when CIC cryoglobulins and complement activation were present (Fig. 4).

In contrast with these observations, in patients with uncomplicated *P. falciparum* infections, complement abnormalities occurred infrequently and to a lesser degree since only 2 of 23 patients had low levels of C4 and 5 of 23 had low levels of C3.

DISCUSSION

The main conclusion of this work is that CIC and in vivo complement activation leading to hypocomplementemia are clearly more common in patients with cerebral complications of *P. falciparum* malaria than in patients without this complication.

Circulating immune complexes (14, 15) have been shown previously to occur in both experimental animals and humans with malaria and to induce pathological consequences through their deposition in various organs, such as the kidney (2, 4, 5, 7-9, 13-16, 27) or the choroid plexus (15). More intensive studies of the complement system are available. Low levels have been reported in monkeys with experimental malaria (3, 10). In humans, after *Plasmodium vivax* infection (19), low levels of CH50 and C4 could be shown to be related to the degree of parasitemia and also to the presence of complement-fixing antibody. Various studies of C levels have also been conducted in humans with *P. falciparum* infection. Whereas Greenwood (11) found no relation between hypocomplementemia and

TABLE 2. *Complement levels in patients with cerebral malaria*[a]

Patient no.	Complement level at following day after onset of symptoms:																	
	6-9						11-15						19-54					
	CH50[b]	C1q	C4[b]	C3	P[b]	B	CH50	C1q	C4	C3	P	B	CH50[c]	C1q	C4	C3[c]	P[c]	B[c]
1	23	100	24	49	54	60	90	140	140	160	76	200	88	80	21	160	200	200
2	<20	44	<10	<10	62	100	60	ND	<10	39	ND	110	125	ND	95	77	ND	120
3	35	ND	14	51	ND	130	100	ND	37	70	ND	140	160	ND	67	107	ND	130
4	28	ND	35	32	ND	81	21	105	<10	67	58	100	ND	ND	ND	ND	ND	ND
5	<20	62	<10	115	42	110	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
6	ND	ND	ND	ND	ND	ND	42	66	41	54	82	100	112	200	200	190	200	165
7	85	200	130	145	92	200	ND	ND	ND	ND	ND	ND	95	200	200	170	135	200
8	20	37	31	39	65	96	ND	185	72	125	195	200	ND	ND	ND	ND	ND	ND
9	ND	200	155	125	60	200	ND	200	80	170	ND	200	ND	200	72	125	98	200
Normal ± 2 SD[d]	65–135	60–135	50–210	68–110	43–150	40–210												

[a] All results are expressed in a percentage of a pool of normal human sera. ND, not done.
[b] Mean values are significantly different from normal as follows: CH50, $P < 0.001$; C4, $P < 0.05$; properdin, $P < 0.005$.
[c] Mean values are significantly different from the period of day 6 to 9: CH50 and properdin, $P < 0.002$; C3, $P < 0.05$.
[d] SD, standard deviation.

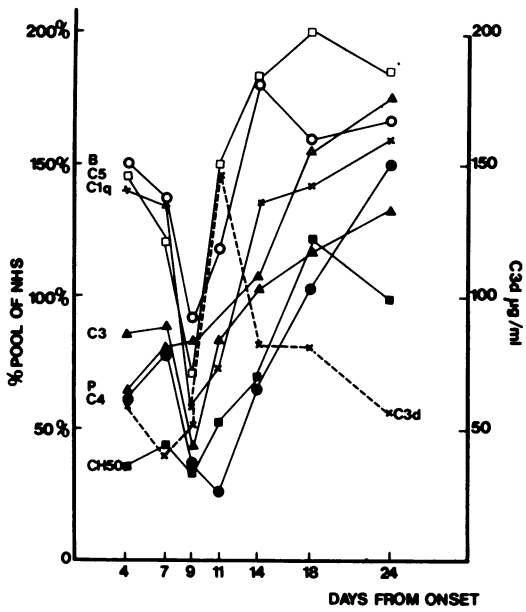

FIG. 3. *Mean blood levels of various complement components and of C3d (ordinate) for the nine patients calculated when available on various days after onset of symptoms (4, 7, 9, 11, 14, 18, 24). Note profound decrease in complement levels associated with raised C3d levels. NHS, normal human serum.*

the severity of clinical symptoms, complement levels observed in the work of Petchclai et al. (23) and Srichaikul et al. (25), as well as C1q disappearance curves, seemed to indicate a relation between C consumption and clinical complications, such as anemia and thrombocytopenia.

Our work, comparing two large groups of patients with *P. falciparum* infection with and without cerebral complications, strongly supports the conclusion that there is a relation between C consumption and clinical complications since hypocomplementemia was considerably more frequent in cerebral malaria. Furthermore, sequential measurements showed that the highest levels of CIC and cryoglobulins coincided with maximum hypocomplementemia and the highest C3d levels. These observations suggest that immune complexes formed at least in part in the circulation were mainly responsible for complement activation. Nevertheless, some degree of hypocomplementemia and an increase in C3d levels were present early in the disease, when CIC and cryoglobulins were present only in small amounts. It is possible that, at this time, hypocomplementemia was induced by a direct complement activation by malarial antigens. Such an activation, previously suggested by

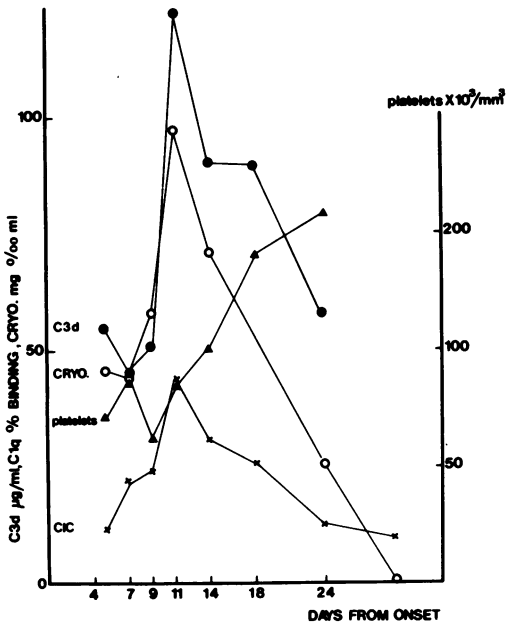

FIG. 4. *Mean blood levels of platelets, circulating immune complexes, cryoglobulin, and C3d (ordinate) for the nine patients calculated when available on various days after onset of symptoms (4, 7, 9, 11, 14, 18, 24). Note that low platelet counts are associated with cryoglobulin, raised CIC, and C3d levels. CRYO, cryoglobulin.*

Neva et al. (19), has not yet been demonstrated with plasmodial antigens, but is known to occur with other parasitic antigens derived from trypanosomes (21), filarias (26), and hydatid cysts (12).

The antigenic composition of CIC could not be established in detail in this work. However, studies of the cryoglobulins showed that they were formed of IgG anti-*P. falciparum*, probably in the form of immune complexes, which could react with IgM, rheumatoid factor. Since there was a good correlation between cryoglobulin levels and C1q-binding activity, and since all three cryoglobulins could bind C1q, it is likely that the C1q-binding material detected was composed at least in part of complexes derived from malarial antigens. Since CIC, cryoglobulins, and complement abnormalities are uncommon in patients with benign *P. falciparum* malaria, it is tempting to postulate that they play a role in the pathogenesis of the cerebral symptoms which occur in some cases of *P. falciparum* malaria. It is interesting in this respect that the degree of coma increased in four of the seven patients who had a secondary increase of C1q-binding material and cryoglobulinemia after quinine therapy was initiated. Indeed at this time, the release of antigens could induce an increased formation of CIC which could be locally deposited in the choroid plexus, as recently found by June et al. in mice infected with *P. berghei* (15). CIC can induce pathological manifestations through pathways which do not involve their deposition in tissues. Indeed, complement activation involves liberation of low-molecular-weight vasoactive peptides, with potential shock-inducing activities. Also, activation of the complement system can affect platelet activation (31) and initiate blood coagluation. The relationship between thrombocytopenia, complement activation, and antibody response to plasmodial antigens has been mentioned by Neva et al. (20) in simians with malaria, and it is interesting to note that thrombocytopenia was present in all of our patients, whereas signs of disseminated intravascular coagulation were found only in 4 of 10 patients. It is thus unlikely that thrombocytopenia is only a consequence of disseminated intravascular coagulation. In five patients, a decrease in platelet count correlated with an increase in the levels of cryoglobulinemia and C1q-binding material. Furthermore, positive IgG direct Coombs tests on platelets were found in four of seven patients (C. Patereau, J. Y. Muller, C. Gibert, P. Verroust, manuscript in preparation). It is therefore possible that the binding of CIC to platelets could be an important step in the development of malarial pathology.

The observations presented in this paper suggest that the intensity of the immune response and the associated complement activation may be important factors in the pathogenesis of malaria. It is interesting to note that in the hamster infected with *P. berghei*, Wright (29) and Wright et al. (30) had shown that neonatal thymectomy or administration of anti-thymocyte serum could almost suppress cerebral hemorrhagic lesions which occur during infection in this animal. It is not possible to state with certainty why some patients with cerebral malaria make an unusually strong immune response. However, it is interesting to note that 8 of our 10 patients with cerebral malaria had previously been in an endemic area and, in two instances, had actually had a malarial infection. It is therefore possible that a first exposure to malarial antigens, uncertain but likely in most of our patients, could lead to a more intense (secondary type) immune response, as compared with a group of patients who had never been in contact with plasmodium antigens before. This in turn could induce larger amounts of CIC, leading to intravascular activation of complement and thrombocytopenia. Such a sequence of events is somewhat similar

to the finding in dengue hemorrhagic shock (6, 24), which occurs only during a second infection and is associated with intense complement activation and CIC.

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