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Distinct pattern of class and subclass antibodies in immune complexes of children with cerebral malaria and severe malarial anaemia

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SUMMARY

Plasmodium falciparum *infection can lead to deadly complications such as severe malaria-associated anaemia (SMA) and cerebral malaria (CM). Children with severe malaria have elevated levels of circulating immune complexes (ICs). To further investigate the quantitative differences in antibody class/subclass components of ICs in SMA and CM, we enrolled 75 children with SMA and 32 children with CM from hospitals in western Kenya and matched them to 74 and 52 control children, respectively, with uncomplicated symptomatic malaria. Total IgG IC levels were always elevated in children with malaria upon enrolment, but children with CM had the highest levels of any group. Conditional logistic regression showed a borderline association between IgG4-containing IC levels and increased risk of SMA (OR = 3·11, 95% CI 1·01–9·56,* $P = 0·05$*). Total IgG ICs (OR = 2·84, 95% CI 1·08–7·46,* $P = 0·03$*) and IgE-containing ICs (OR = 6·82, OR 1·88–24·73,* $P \leq 0·01$*) were associated with increased risk of CM. These results point to differences in the contribution of the different antibody class and subclass components of ICs to the pathogenesis of SMA and CM and give insight into potential mechanisms of disease.*

Keywords *cerebral malaria, circulating immune complexes, malaria, anaemia*

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INTRODUCTION

Plasmodium falciparum is the most lethal malaria parasite of humans. Most deaths occur due to complications such as severe malaria-associated anaemia (SMA) and cerebral malaria (CM). Malaria infection leads to development of malaria-specific antibodies and to antigenemia (1). Consequently, immune complexes (ICs) form during malaria infection (2,3). ICs can activate complement and deposit in tissues stimulating an inflammatory response (4). Therefore, some investigators have suggested a possible role for ICs in the pathogenesis of SMA and CM (5,6).

In a previous study, we showed increased levels of ICs in children with severe malaria but no clear differences between children with SMA and CM (6). Because immunoglobulin classes and subclasses differ in their ability to interact with Fc receptors on macrophages, activate the complement cascade and stimulate pro-inflammatory cytokines (7–9), we reasoned that ICs from patients with different forms of clinical malaria may differ in the amounts of antibody class and subclass components they contain. Therefore, in the present study, we investigated the association between the amount of antibody class and subclass components of ICs and the risk of SMA and CM.

MATERIALS AND METHODS

Study design and patient population

Participants were recruited under a human use protocol approved by the Human Use Research Committee, the Walter Reed Army Institute of Research, Silver Spring, MD, USA, and the National Ethics Review Committee of the Kenya Medical Research Institute, Nairobi, Kenya. Informed consent was obtained from all parents or guardians. The study had a matched case-control design. In comparison to our previous studies, we modified the case definition for

SMA cases from Hb ≤ 5 g/dL to Hb ≤ 6 g/dL to increase the probability of finding red cells with complement (C3) deposition in support of other studies (10). Thus, SMA cases, defined as children with asexual *P. falciparum* parasitemia by Giemsa-stained thick and thin blood smear and Hb ≤ 6 g/dL, were recruited from the paediatric ward of the Nyanza Provincial General Hospital (NPGH), Kisumu, Kenya, where malaria is holoendemic. Because CM is uncommon in this area, CM cases were recruited from the paediatric ward of the Kisii District Hospital (KDH), as well as from the NPGH. KDH is located in the highlands of western Kenya where transmission is seasonal and consequently receives many more CM cases than the NPGH (11). CM was defined as asexual *P. falciparum* parasitemia by Giemsa-stained blood smear and a Blantyre coma score of ≤ 2 (12), lasting at least 30 min if there was a history of convulsions. Symptomatic uncomplicated malaria controls matched by gender and age ± 2 months were assigned to each case at a case:control ratio of 1 : 1 for SMA and 1 : 1–2 for CM, and were identified from the outpatient clinic of the same hospital where the corresponding case was recruited. Controls were defined as children with a normal mental status, a Hb > 6 g/dL, a Giemsa-stained blood smear positive for asexual *P. falciparum* and an axillary temperature = 37·5°C. In the absence of fever, we required two of the following signs or symptoms: nausea/vomiting, irritability, poor feeding, myalgias or headache. General exclusion criteria also included evidence of concomitant serious infections (i.e. meningitis excluded by lumbar puncture when indicated, pneumonia, sepsis), chronic illness or a history of blood transfusion in the 3 months preceding enrolment to avoid the influence of donor erythrocytes in our measurements.

All study participants were evaluated in a standardized fashion at enrolment (visit 1) and at follow-up (visit 2) 2 months later. If a child failed to return for follow-up, a field worker travelled to his/her last known domicile to determine his/her status. During follow-up, a blood sample was obtained once it was confirmed that the child was asymptomatic and free of parasitemia. If malaria persisted at the first follow-up visit, the child was re-treated and re-evaluated 2 weeks later. Inpatient treatment for malaria consisted of IV quinine and outpatient therapy was with artemether/lumefantrine (13).

Collection and processing of blood samples

Giemsa-stained thick and thin blood smears were prepared from capillary blood obtained by finger prick. A 2·5-mL sample of EDTA-anticoagulated venous blood was obtained at enrolment and 5 mL at follow-up. Following measurement of haemoglobin levels, the EDTA-anticoagulated blood was centrifuged and the plasma was stored at –70°C until later use.

Measurement of circulating ICs

The methodology for measurement of total IgG ICs was described in detail before (6). Briefly, we used a C1q-based ELISA assay. Wells of Immulon II HB 96-well plates (Thermo Labsystems, Helsinki, Finland) were coated overnight with 10 μg/mL of C1q (Sigma-Aldrich, St Louis, MO). Aggregated IgG, prepared from purified human IgG (Sigma-Aldrich) by heating at 63°C for 30 min followed by size fractionation over a Sephacryl S-300 70 × 2·6 cm column (Amersham Pharmacia Biotech, Piscataway, NJ), served as a standard. Control and test plasma were diluted 1 : 50 in dilution buffer (PBS/0·5% boiled casein, 0·025% Tween, 0·01% Thimerosal, 20 μg/mL phenol red) and 100 μL added to duplicate wells followed by incubation for 1 h at room temperature. The wells were emptied and washed four times with wash buffer (PBS, 0·025% Tween). Total IgG was detected by using horse radish peroxidase (HRP)-conjugated goat antihuman IgG (Kirkegaard & Perry, Baltimore, MD) at dilution of 1 : 3000. For detection of antibody subclasses we used biotin-labelled monoclonal antibodies against human IgG1 (Clone 8c/6-39, Sigma-Aldrich) at a dilution of 1 : 8000, IgG2 (Clone G18-21, Becton-Dickinson, Brussels, Belgium) at a dilution of 1 : 1000, IgG3 (Clone HP6047, Zymed Laboratories, South San Francisco, CA) at a dilution of 1 : 2000 and IgG4 (Clone JDC-14, Becton-Dickinson) at a dilution of 1 : 4000. After incubation, the wells were emptied and washed four times with wash buffer. HRP-conjugated streptavidin (Sigma-Aldrich) was diluted 1 : 6000 in dilution buffer and 100 μL was added to each well followed by 30 min incubation at room temperature. After washing four times, 200 μL of ABTS substrate (Kirkegaard & Perry) was added to each well and incubated for 30 min followed by measurement of the $OD_{415\,nm}$. Total IgG and subclass IC levels were expressed as micrograms of aggregated human IgG equivalent per ml (μg AHG /mL). Calculation of each subclass concentration in the standard was based on the approximate percentage concentration of each IgG subclass (14). Positive and negative control samples were used in every plate. Plate-to-plate variation was controlled by normalizing to the positive control sample by using the following formula

$$ICc(\mu gAHGEq/mL) = ICuc \times Cm/Cp$$

where ICc is the corrected IC level of the sample, ICuc is the uncorrected IC level, Cm is the average concentration of the IC for the positive control for the study and Cp is the

concentration of the positive control for the plate in which the sample was tested.

To detect IgA and IgE-containing ICs, a sandwich ELISA assay was used. In order to rule out detection of free Igs, ICs were precipitated from plasma by adding 100 µL of 4% PEG (w/v) solution (PEG 6000, Fluka, St Louis, MO) to 100 µL of plasma diluted 1 : 5 in borate buffer pH 8·5 (Pierce, Rockford, IL). This was mixed well and incubated overnight at 4°C followed by centrifugation at 5000 r.p.m. for 10 min at 4°C. The IC precipitate was washed twice with 200 µL 2% PEG solution. IC was then re-suspended in 100 µL borate buffer pH 8·5 and stored frozen until used. Wells of Immulon II HB 96-well plates (Thermo Labsystems) were coated with 100 µL of goat antihuman IgE antibody or antihuman IgA (Kirkegaard & Perry) diluted 1 : 2000 in dilution buffer and incubate overnight at 4°C. The plate was washed four times with wash buffer (0·25% Tween 20 in PBS pH 7·4) and then blocked for 1 h with 200 µL of blocking buffer (PBS, 0·5% boiled casein, 1% Tween, 0·01% Thimerosal, 20 µg/mL phenol red). After four washes, 100 µL of IgE or IgA standard (Sigma-Aldrich) at various dilutions, control samples or PEG-precipitated test samples diluted 1 : 50 in dilution buffer was added to duplicate wells and incubated for 1 h at room temperature. This was followed by four washes with wash buffer and addition of 100 µL of HRP-conjugated goat antihuman IgE or IgA (Sigma-Aldrich) diluted 1 : 500 or 1 : 3000, respectively, in dilution buffer and incubation for 1 h at room temperature. Colour development, absorbance measurement and normalization were as described for IgG above. The quantities were expressed as µg of human immunoglobulin Ig/mL.

Statistical analysis

Statistical analysis was performed using SPSS for windows version 11·5 (SPSS Inc., Chicago, IL) software package. Mean IgG, IgG subclass, IgE and IgA-containing IC levels were compared between cases and controls by multivariate ANOVA with matching and adjustment for home districts. Because most multiple comparison procedures include more comparisons than was of interest *a priori*, increasing the probability of a Type II error, we used a primary analysis that consisted of pairwise comparisons using least squares. A secondary analysis consisted of correction for multiple comparisons using Tukey's honestly significant difference test. Comparison of IC levels between SMA and CM cases at enrolment and follow-up was done using Mann–Whitney *U*-test due to unequal variances. Multivariate conditional logistic regression controlling for district of residence, and haemoglobin level where appropriate, was carried out to determine the association of antibody class/subclass and severity of malaria at enrolment and follow-up. To make meaningful associations, the IC levels were converted to standard deviation (SD) units by dividing by SD of antibody class or subclass for each cohort. All tests were two-tailed with $\alpha \leq 0{\cdot}05$.

RESULTS

Demographics

Seventy-five SMA and 32 CM cases were enrolled and matched to 74 and 52 symptomatic uncomplicated malaria controls, respectively. The demographics and clinical characteristics of the study participants were recently reported (10). The mean age (SD) for SMA cases was 16·9 (13·7) months and that of their controls was 16·8 (13·3) months. The same parameters for CM cases and their controls were 33·1 (19·2) and 33·6 (16·4) months, respectively. There were no significant differences between cases and controls in the districts of origin, ethnic origin or parasite density (10). There were also no significant differences in the parasite densities between CM and SMA cases at enrolment (10). Sixty-five SMA cases and 61 of their uncomplicated malaria controls presented for follow-up. There were five deaths among patients with SMA (6·7% mortality), all of which occurred during the initial hospitalization. There was one death among the SMA controls (1·3% mortality), which was due to an episode of severe diarrhoea. Twenty-seven CM cases and 44 of their controls presented for follow-up. One in-hospital death occurred among CM cases recruited at KDH (4·2% mortality) and no deaths occurred among CM cases at NPGH. Most of the missed follow-up visits were due to the parents declining further participation in the study or moves out of the study area.

IC levels

Comparison of IC levels between enrolment and follow-up

Table 1 summarizes the IC levels among cases and controls at the time of enrolment (visit 1) and follow-up (visit 2). The number of samples actually tested was slightly smaller than the total number of cases and controls due to insufficient volume of some samples. ICs were nearly always higher at enrolment than at follow-up. This is consistent with previous observations of increased level of ICs during malaria infection (6). However, unlike our previous observations (6), the decline in IC levels with treatment was not statistically significant for SMA cases. The reasons for this are unclear. One possible explanation is the expansion of the SMA case definition from the traditional Hb ≤ 5 g/dL to Hb ≤ 6 g/dL. However, a subgroup analysis of children with Hb ≤ 5 g/dL revealed similar results (data not shown).

Table 1 Mean (SD) IC levels among cases and controls at enrolment and follow-up

	SMA cases				SMA controls				SMA cases vs. controls			
			Visit 1 vs. visit 2				Visit 1 vs. visit 2		Visit 1		Visit 2	
IC type[a]	Visit 1 $N = 74$	Visit 2 $N = 63$	Mean difference 95% CI	P	Visit 1 $N = 70$	Visit 2 $N = 59$	Mean difference 95% CI	P	Mean difference 95% CI	P	Mean difference 95% CI	P
IgG	4·1 (3·5)	3·5 (5·6)	–0·9 to 1·5	0·61	3·9 (1·3)	2·7 (3·1)	0·0 to 2·5	0·04	–1·1 to 1·4	0·82	–0·2 to 2·5	0·11
IgG1	1·0 (1·8)	0·6 (1·8)	–0·3 to 0·6	0·49	1·0 (1·4)	0·6 (1·0)	–0·01 to 0·85	0·06	–0·5 to 0·3	0·73	–0·3 to 0·7	0·41
IgG2	0·1 (0·2)	0·1 (0·2)	–0·04 to 0·04	0·94	0·2 (0·2)	0·1 (0·2)	–0·01 to 0·07	0·12	–0·07 to 0·01	0·14	–0·04 to 0·04	0·99
IgG3	0·5 (0·6)	0·4 (0·7)	–0·1 to 0·3	0·54	0·4 (0·7)	0·3 (1·1)	–0·1 to 0·4	0·27	–0·2 to 0·2	0·85	–0·2 to 0·3	0·52
IgG4	0·2 (0·3)	0·1 (0·2)	0·0 to 0·1	0·04	0·1 (0·2)	0·1 (0·1)	–0·01 to 0·11	0·12	–0·02 to 0·11	0·14	–0·04 to 0·10	0·36
IgA	4·0 (3·0)	3·4 (2·8)	–0·6 to 2·1	0·30	5·3 (6·3)	4·0 (4·1)	0·2 to 3·0	0·02	–3·0 to –0·2	0·03	–2·2 to 0·9	0·38
IgE	0·2 (0·2)	0·2 (0·1)	–0·03 to 0·05	0·53	0·2 (0·2)	0·2 (0·1)	–0·02 to 0·06	0·42	–0·06 to 0·02	0·42	–0·06 to 0·03	0·58
	CM cases				CM controls				CM cases vs. controls			
			Visit 1 vs. visit 2				Visit 1 vs. visit 2		Visit 1		Visit 2	
	Visit 1 $N = 32$	Visit 2 $N = 25$	Mean difference 95% CI	P	Visit 1 $N = 49$	Visit 2 $N = 40$	Mean difference 95% CI	P	Mean difference 95% CI	P	Mean difference 95% CI	P
IgG	8·5 (4·7)	4·9 (3·4)	1·2 to 6·2	< 0·01[b]	6·6 (4·0)	3·3 (2·3)	1·3 to 5·2	< 0·01[b]	0·2 to 5·0	0·03	–0·3 to 4·8	0·09
IgG1	3·2 (2·4)	1·3 (1·8)	0·2 to 3·2	0·03[b]	2·8 (3·4)	0·4 (0·5)	1·2 to 3·6	< 0·01	–0·7 to 2·1	0·33	–0·1 to 3·0	0·07
IgG2	0·6 (1·0)	0·2 (0·3)	0·0 to 0·5	0·02[b]	0·3 (0·3)	0·2 (0·3)	–0·1 to 0·3	0·25	–0·01 to 0·46	0·07	–0·2 to 0·3	0·72
IgG3	0·8 (1·3)	0·3 (0·4)	0·1 to 1·0	0·01[b]	0·6 (0·7)	0·2 (0·3)	0·2 to 0·8	< 0·01[b]	–0·2 to 0·6	0·26	–0·2 to 0·6	0·42
IgG4	0·5 (0·8)	0·2 (0·2)	0·0 to 0·5	0·04	0·3 (0·4)	0·1 (0·1)	–0·1 to 0·3	0·20	0·1 to 0·5	0·02	–0·1 to 0·3	0·25
IgA	7·8 (7·9)	5·3 (4·0)	–1·6 to 5·2	0·30	7·6 (7·0)	6·1 (4·8)	–1·3 to 4·1	0·30	–3·4 to 3·0	0·91	–1·3 to 4·1	0·74
IgE	0·2 (0·2)	0·1 (0·1)	0·0 to 0·1	0·05	0·1 (0·1)	0·1 (0·1)	–0·05 to 0·04	0·98	0·01 to 0·11	0·02[b]	–0·1 to 0·1	0·98

[a]Units are in µg of aggregated human IgG equivalent (µg AHG)/mL for total IgG and subclasses. For IgA and IgE, units are in µg of human Ig/mL.
P-values were obtained by using multivariate analysis of variance with matching with adjustment for home districts.
[b]*P*-values were significant after adjustment for multiple comparisons using Tukey's honestly significant difference test.

Only IgG4-containing ICs were higher among SMA cases than controls in the primary analysis but not after correcting for multiple comparisons. CM cases, on the other hand, had relatively high IC levels at enrolment for most IgG subclasses and IgE which declined significantly upon follow-up. The only significant difference between CM cases in KDH and their counterparts enrolled in NPGH was that at follow-up CM cases enrolled in KDH had lower IgA-containing ICs than in NPGH, mean (SD) for KDH = 4·4 (5·2) and for NPGH = 10·1 (7·1), $P = 0·01$. CM controls enrolled in KDH differed from those enrolled in NPGH in that the latter had higher levels of IgG2 and IgE-containing ICs during follow-up, IgG2 KDH mean (SD) = 0·26 (0·27) and NPGH mean (SD) = 0·09 (0·12) $P = 0·03$, and for IgE KDH mean (SD) = 0·13 (0·07) and NPGH mean (SD) = 0·09 (0·03) $P = 0·04$.

Comparison of IC levels between cases and controls

There was no statistically significant difference in IC levels between SMA cases and their controls except that IgA-containing ICs were higher in controls than in cases at enrolment, but this difference disappeared after adjustment for multiple comparisons (Table 1). On the other hand, CM cases had higher total IgG, IgG4 and IgE-containing IC levels than their controls at enrolment. The strongest difference was with IgE-containing ICs which held up to adjustment for multiple comparisons. All these differences disappeared at follow-up.

Comparison of IC levels between SMA and CM cases

Table 2 summarizes the comparison between SMA and CM cases at enrolment and follow-up. CM cases had higher levels of all ICs except for IgG3 and IgE than SMA cases at

Table 2 Comparison of IC levels between SMA and CM cases at enrolment and follow-up

IC type[a]	Visit 1[b]			Visit 2[b]		
	SMA ($N = 74$)	CM ($N = 32$)	P[c]	SMA ($N = 63$)	CM ($N = 25$)	P[c]
IgG	2·9 (0·4–17·5)	6·1 (1·2–36·8)	< 0·01	2·1 (0·5–41·6)	3·6 (0·5–16·4)	0·02
IgG1	0·5 (0·0–13·3)	0·9 (0·2–16·2)	< 0·01	0·2 (0·0–13·6)	0·5 (0·0–5·9)	0·01
IgG2	0·1 (0·0–0·6)	0·3 (0·0–4·6)	< 0·01	0·0 (0·0–0·7)	0·1 (0·0–1·5)	0·27
IgG3	0·3 (0·0–3·7)	0·4 (0·0–7·0)	0·55	0·2 (0·0–4·4)	0·1 (0·0–1·6)	0·17
IgG4	0·1 (0·0–1·4)	0·2 (0·0–4·5)	< 0·01	0·0 (0·0–1·3)	0·2 (0·0–0·9)	< 0·01
IgA	3·3 (0·3–13·5)	5·2 (0·1–32·2)	0·01	2·8 (0·3–13·8)	4·3 (0·5–20·4)	0·01
IgE	0·2 (0·0–0·6)	0·2 (0·0–1·2)	0·80	0·1 (0·0–0·6)	0·1 (0·0–0·3)	0·49

[a]Units are in μg of aggregated human IgG equivalent (μg AHG)/mL for IgG and subclasses. For IgA and IgE units are in μg of human Ig/mL. [b]Values represent medians and ranges in parenthesis. [c]P-values obtained using Mann–Whitney U-test.

Table 3 Conditional logistic regression for immune complex levels at enrolment

IC type	Severe anaemia[a]			Cerebral malaria[b]		
	OR	95% CI OR	P	OR	95% CI OR	P
IgG	0·46	0·14–1·49	0·20	2·84	1·08–7·46	0·03
IgG1	1·28	0·62–2·65	0·51	0·86	0·71–1·03	0·10
IgG2	0·56	0·27–1·15	0·12	1·66	0·65–4·23	0·29
IgG3	1·71	0·74–3·94	0·21	0·85	0·46–1·55	0·59
IgG4	3·11	1·01–9·56	0·05	0·81	0·54–1·21	0·30
IgA	0·74	0·50–1·08	0·12	0·30	0·11–0·84	0·02
IgE	0·88	0·43–1·81	0·732	6·82	1·88–24·73	< 0·01

[a]Adjusted for home districts. [b]Adjusted for home districts and haemoglobin levels.

Table 4 Conditional logistic regression for immune complex levels at follow-up

IC type	Severe anaemia[a]			Cerebral Malaria[a]		
	OR	95% CI OR	P	OR	95% CI OR	P
IgG	28·69	2·75–299·75	< 0·01	1·26	0·24–6·64	0·78
IgG1	3·38	0·16–72·94	0·44	2·44	1·25–4·75	0·01
IgG2	0·21	0·05–0·96	0·04	0·11	0·01–1·17	0·07
IgG3	4·65	0·04–538·69	0·53	0·59	0·05–6·76	0·67
IgG4	71·80	0·50–10349·65	0·09	0·77	0·17–3·56	0·74
IgA	0·64	0·64–1·26	0·19	0·07	< 0·01–1·04	0·05
IgE	0·23	0·06–0·89	0·03	0·47	0·09–2·43	0·37

[a]Adjusted for home districts and haemoglobin levels.

enrolment. These differences persisted during follow-up except for IgG3.

Conditional logistic regression

We carried out multivariate conditional logistic regression to identify the subclass and class of antibody-containing ICs that independently were most strongly associated with severe malaria. Table 3 summarizes the findings upon enrolment. IgG4-containing ICs were the only independent predictors of SMA. Although the effect was large, the level of significance was borderline ($P = 0·05$). On the other hand, total IgG and IgE-containing ICs were most strongly associated with the development of CM whereas IgA-containing ICs seemed to be protective.

Assuming that the condition of the children at follow-up was similar to their baseline state, we tested for the association between class and subclass IC levels and severe malaria during follow-up (Table 4). Total IgG IC levels were associated with an increased risk of SMA whereas IgE-containing ICs were protective against SMA during follow-up (Table 4). IgG1-containing ICs were associated with increased risk of CM, and there was a trend of association between IgG2 and IgA-containing ICs and decreased risk of CM.

DISCUSSION

Plasmodium falciparum malaria infection leads to generation of ICs which can deposit on endothelial surfaces and activate the complement cascade, resulting in the production of a number of pro-inflammatory mediators (15). Also, by cross-linking Fc receptors on effector cells such as macrophages and monocytes, ICs can stimulate the production of pro-inflammatory cytokines (16) that have been proposed to have a role in the pathogenesis of severe malaria (17). Despite the accumulating evidence linking ICs to the pathogenesis of malaria (2,5,6,18–20), no clear pattern of association between antibody class or subclass and the clinical form of severe malaria has emerged. Therefore, the main objective of this study was to determine the composition

of ICs in terms of antibody class/subclass and to relate this to the severity of *P. falciparum* malaria.

In general, IC levels were high at enrolment and diminished in all groups in response to malaria treatment (Table 1). This is consistent with our previous observations (6) and suggests that the initial malaria infection was responsible for the elevated IC levels. However, unlike our previous observations (6), the decline in IC levels with treatment was not statistically significant for SMA cases. We believe the explanation lies in the differences in the length of follow-up between the two studies. The former study had a follow-up period of 4 months as opposed to 2 months in the present study. Therefore, children with SMA may have a lower capacity to clear ICs from circulation since they require a longer period of time to do so. Interestingly, we have reported that red cells of children with SMA have acquired deficiencies in CR1/CD35 (10,21,22), a key complement receptor that, among other functions, binds opsonized ICs from circulation. Red cells carry ICs bound via CR1 to the liver and spleen where they are removed (23). Therefore, the decreased ability of children with SMA to clear ICs may be due to the deficiency of red cell CR1.

Children with CM had higher levels of total IgG ICs than their controls and children with SMA (Tables 1 and 2). This is consistent with our previous findings and the findings of others (5,6). When compared to their controls, the most significant difference in IC subclass antibody composition was higher level of IgG4-containing ICs in CM cases at enrolment. In addition, levels of IgE-containing ICs were higher in CM cases than in their controls. In the conditional logistic regression model, which controlled for the effects of all IC classes and subclasses, total IgG and IgE-containing IC levels were the only independent predictors of CM at enrolment (Table 3), while IgA, normally considered to have a role in mucosal immunity, was protective. Other investigators have reported an association between total IgE levels and CM and other forms of severe malaria (24,25). The finding of IgG and IgE deposits in brain capillaries of CM fatalities containing sequestered parasitized red cells is in support of the role of ICs in the pathogenesis of CM (19). It has been proposed that these ICs can lead to local overproduction of TNF-α, a cytokine that has been implicated in the pathogensis of CM (17), from monocytes by cross-linking their Fc receptors (26). This could be achieved by the interaction of ICs carried on red cells sequestered in brain capillaries with Fc receptors on monocytes/macrophages and endothelial cells (27–29). The higher the levels of IgG ICs, the higher the load of ICs on red cells and the greater likelihood of an interaction between red cells and macrophages.

On the other hand, except for high levels of IgG4-containing ICs, children with SMA did not differ significantly from their controls at enrolment (Table 1). This observation was confirmed by the logistic regression analysis which showed that the presence of IgG4-containing ICs was the only independent predictor of SMA at enrolment although the statistical significance was borderline ($P = 0{\cdot}05$) (Table 3). The mechanism by which IgG4-containing ICs may increase the risk of SMA is not immediately clear. Although IgG4 is common on the surface of red cells from patients with malaria and in patients with autoimmune haemolytic anaemia (30,31), it is known to be noncytophilic, meaning that it is a poor inducer of phagocytosis (8,32). In addition, IgG4 is a poor activator of the complement cascade and a poor inducer of pro-inflammatory cytokines upon binding to FcγRI (CD64) receptors on macrophages (7,9). A number of studies have also found an association between high levels of total IgG4 or IgG4 antimalarial antibodies and increased susceptibility to clinical malaria attacks or severe malaria (33,34). Conversely, an association between IgG1 and/or IgG3 and protection against uncomplicated and complicated malaria has been observed in several studies (35–39). The basis for the protection of IgG1 and IgG3 subclass antibody has been attributed to their greater opsonophagocytic activity and their complement activation capacity (8,32). IgG4 has been shown to inhibit the IgG1and IgG3-mediated opsonization of infected erythrocytes (8). Therefore, it is possible that the basis of the association between IgG4-containing ICs and increased susceptibility to SMA may be simply the fact that the antibody response is biased towards a nonprotective subclass or a subclass that blocks protective antibody responses leading to an increase in the risk of repeated infections. The incidence of SMA is highest in areas of the world where there is intense transmission and presumably the greatest likelihood of repeated infections.

Assuming that the condition of the participants at follow-up is a good reflection of their baseline between malaria attacks, we felt that studying the relationships between ICs and severe malaria during this visit could be enlightening. Although participants during follow-up were negative for malaria by microscopy and did not exhibit any clinical signs of disease, it seems reasonable to assume that the ICs detected at this time were residual from the previous malaria infection, and/or derived from continuous antigenic malaria challenge and other infections during the intervening follow-up period. These intervening events may be equally important in determining who ultimately develops malaria complications. Whereas during acute malaria total IgG IC levels were associated with protection against SMA (Table 3), these seemed to be deleterious at follow-up (Table 4). This observation is consistent with our argument that continuous antigenic stimulation and IC formation can lead to complement activation and are key for the development of SMA (40). On the other hand, the presence

of IgG2 and IgE-containing ICs during follow-up seemed to be protective against SMA. Antimalarial IgE has been observed to be protective from subsequent infections in a longitudinal study (41). The mechanism for the association between IgG2-containing ICs and decreased risk of SMA is not clear but in some studies antimalarial IgG2 has been associated with resistance against malaria (34). These data again suggest that the avoidance of recurrent malaria infections is important in preventing SMA. The association between IgG or IgE-containing ICs and CM disappeared during follow-up. Instead, we were surprised to see borderline protective effect from noncytophilic and noncomplement activating ICs (IgG2 and IgA) while the presence of IgG1-containing ICs was found to be a risk factor. The role of IgA in protection from CM deserves further investigation.

In summary, our findings suggest that although both SMA and CM are characterized by high levels of ICs, the class and subclass make-up of these ICs as well as the role that they play in each may be distinct. While CM is characterized by high levels of IgG and IgE-containing ICs that may serve to stimulate local production of pro-inflammatory cytokines in sequestered brain capillaries, SMA is characterized by persistent lower level IgG ICs in the intervening period between malaria attacks, the subclass make-up of which may interfere with adequate immune response and allow repeated infections.

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