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High levels of anti-phospholipid antibodies in uncomplicated and severe *Plasmodium falciparum* and in *P. vivax* malaria

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SUMMARY

The majority (75%) of adult patients with uncomplicated *Plasmodium falciparum* and *P. vivax* malaria are positive for anti-phospholipid antibodies (aPLA) as demonstrated by ELISA using a panel of anionic and cationic phospholipids. The highest IgG and IgM binding was to the anionic phospholipids, phosphatidylserine (PS), phosphatidic acid (PA) and cardiolipin (CL), but excluding phosphatidylinositol (PI) to which only low antibody levels were found. Comparison of the mean IgG and IgM aPLA showed a trend for anti-PA > CL > PS > PC > PE > PI. Anti-PI levels were compared in two groups of African children, one group with non-severe and the other with severe (cerebral) falciparum malaria. Children with cerebral disease had significantly lower IgM anti-PI. The results are discussed with the view that serum-derived aPLA may have a role in 'anti-disease' immune responses. Their possible role in the opsonization and phagocytosis of parasitized erythrocytes and in thrombocytopenia is also considered.

Keywords malaria anti-phospholipid antibodies 'anti-disease' immunity tumour necrosis factor

INTRODUCTION

Antibodies to phospholipids (aPLA) occur in certain autoimmune diseases, notably systemic lupus erythematosus (SLE) and the primary anti-phospholipid syndrome (aPLS [1]). Raised levels of aPLA are also seen as a frequent reaction to a number of infections which include syphilis, African trypanosomiasis, Lyme disease, infectious mononucleosis and AIDS [2]. The stimulus for the production of these antibodies remains unknown.

aPLA represent a diverse group of antibodies which may have important pathophysiological consequences *in vivo*. Most patients with SLE or aPLS have characteristically high titres of aPLA (as detected by solid-phase immunoassay) to anionic phospholipids (particularly cardiolipin), with a predominance of antibodies of the IgG isotype in individuals with clinical symptoms such as thrombosis, thrombocytopenia and recurrent fetal loss [3].

The present study is the first detailed description, to our knowledge, of aPLA in malaria, and compares levels, specificity and immunoglobulin class distribution of aPLA in uncomplicated *Plasmodium falciparum* and *P. vivax* infections in adults, and in children with non-severe *versus* severe (cerebral) falciparum malaria. The investigation was prompted by the possibility that antibodies to certain phospholipids may have beneficial effects in the infection by both enhancing phagocytosis of parasitized erythrocytes (where membrane asymmetry is lost) and by binding to parasite-derived phospholipid antigens known to have tumour necrosis factor (TNF)-stimulating properties and effects on glucose metabolism [4,5]. An understanding of the specificity of aPLA which may be capable of inhibiting these functions is clearly of importance, and may have practical implications. The results lend support to the existence of a specific immunologic response to malaria-derived phospholipid antigens.

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PATIENTS AND METHODS

Patients and plasma

All blood samples were collected into ethylenediaminotetracetic acid (EDTA, 1 mg/ml) and the plasma stored at −70°C. Patients and donors fell into one of five groups, as follows:

Group A: a negative control group comprising 30 normal healthy adult donors (17 Caucasian (age range 25–48 years), 13 Negroid (age range 21–28 years plus two East African negro children age matched with those of Groups D and E below)).

Group B: a positive control group of 10 patients with SLE. The plasma samples were kindly donated by Dr S. Bashir, Royal London Hospital, UK.

Group C: plasma obtained from 28 adult patients (10 Caucasian, 10 Negroid, eight Asian) attending The Royal London Hospital

for treatment of uncomplicated non-severe malaria. Nineteen patients had *P. falciparum* with parasitaemias ranging from < 1% to 5%, and nine patients had *P. vivax* all with parasitaemias < 1%. The period of time between onset of symptoms of malaria and treatment ranged from 2 days to 2 weeks. None had any known autoimmune or other infectious disease.

Group D: plasma from seven East African negro children (age 1–5 years) with non-severe *P. falciparum* malaria (parasitaemia range 180 200–600 000/μl). The children were residents of areas hyperendemic for malaria. A single blood sample was taken on admission to hospital and before any antimalarial chemotherapy. The separated plasmas were transported in liquid nitrogen to London for testing.

Group E: plasma from seven East African negro children, matched with those in Group D for age, ethnic group and area of residence, defined as having cerebral malaria with an admission coma score from 0 to 4 on a modified Glasgow scale [6]. Parasitaemias ranged from 155 000 to 1 036 000/μl. Blood samples were obtained and processed as in Group D.

Parasitological examination

Thick and thin blood films were stained with Giemsa and parasitaemia calculated either as percentage of erythrocytes parasitized (Group C) or number of parasites per 1 μl of blood (Groups D and E).

ELISA for aPLA

The following six phospholipids were used (all from Sigma): cardiolipin (CL) in methanol, phosphatidic acid (PA) and phosphatidylserine (PS) in 95:5 chloroform/methanol, phosphatidylinositol (PI) containing primarily linoleic and palmitic acids in 9:1 chloroform/methanol, phosphatidylcholine (PC), and phosphatidylethanolamine (PE) in chloroform.

The ELISA was a slight modification of that previously described [7,8]. Polystyrene 96-well flat-bottomed microtitre plates for ELISA (Sigma) were coated for 48 h at 4°C with 50 μl/well of either a 50 μg/ml solution of the individual phospholipids (PA, CL, PS, PE in 1:4 chloroform/methanol; PI in 9:1 chloroform/methanol) or a mixture of the anionic phospholipids (PA, CL, PS but not including PI), or cationic phospholipids (PC and PE) each at a concentration of 50 μg/ml in methanol. Evaporation of the solvent solution occurs during this period and the lipid antigens adhere strongly to the hydrophobic polystyrene surface.

The plates were blocked, without prior washing, by incubation with 100 μl of PBS/10% fetal calf serum (PBS/FCS) for 1 h at 20°C. In order to reduce non-specific adsorption of immunoglobulin (particularly IgM) to the plates [7], all plasma samples were diluted 1:100 with PBS/FCS and 100 μl added to replicate antigen-coated and control wells and left for 2 h at 20°C. After washing three times with PBS, 100 μl of peroxidase-conjugated goat anti-human IgG or IgM (Sigma) were added at a 1:2000 dilution in PBS/FCS with additional 3% polyethylene glycol to speed up the reaction and to improve sensitivity. Following incubation for 1 h at room temperature, plates were washed and peroxidase activity measured using H_2O_2 and ortho-phenylenamidiamine tablets (Dako) and the optical density read at 492 nm using a BioRad microplate reader. Each plate contained patient, negative (normal individuals) and positive (SLE) control samples.

Anti-phospholipid antibody levels were expressed as number of s.d. above the mean for 30 normal controls; levels > 2 s.d. above this mean were considered positive.

Statistical analysis

Because of the skewed distribution of aPLA, non-parametric tests were applied with the Kruskal–Wallis analysis of variance and the Mann–Whitney *U*-test used to determine significance. Correlations were evaluated using Spearman's rank correlation test and $P < 0.05$ considered to indicate statistical significance.

RESULTS

Isotype distribution of aPLA in uncomplicated P. falciparum *and* P. vivax *malaria*

Of the 28 cases of malaria, 21 (75%) had aPLA of one or both isotypes. A greater percentage of *P. falciparum* patients (84%) were positive than were *P. vivax* patients (67%). More *P. falciparum* cases had both IgG and IgM aPLA (63%) than did those with *P. vivax* (44%), but almost the same percentage of falciparum and vivax patients (33% and 32% respectively) had IgG or IgM alone.

There was no negative or positive correlation between IgG and IgM aPLA levels for all malarias ($r = 0.292$, $P = 0.14$); for *P. falciparum* alone ($r = 0.134$, $P = 0.59$); or for *P. vivax* alone ($r = 0.348$, $P = 0.35$).

No significant difference was observed between aPLA levels of the 17 control adult Caucasian donors, the 13 Negroid normal adult donors and the two East African normal negro children (data not shown).

PL specificity of aPLA

Specificity was examined by measuring reactions to a panel of anionic and cationic PL. The highest levels of IgG and IgM aPLA binding were to the anionic PL, PS, PA and CL, but excluding PI to which only low levels were found in all malaria samples (Fig. 1a–d). Comparison of the mean IgG and IgM aPLA showed a trend for anti-PA > CL > PS > PC > PE > PI.

For *P. falciparum* infections, the most frequently encountered specificity considered positive (that is > 2 s.d. above the normal mean) was IgG aPS (89%) and the lowest IgG aPI (16%). Only a few *P. vivax* patients were positive for IgG and IgM aPI (33% and 23% respectively), but 100% were positive for IgG aPA (Table 1).

Within each infection group, *P. falciparum* or *P. vivax*, there was no statistically significant difference between the mean of all s.d. above the normal mean for IgG and IgM for each specificity, with the exception of the mean IgG aPA and aPI ($P < 0.05$).

Antibodies to PI in adult patients with primary uncomplicated P. falciparum *malaria*

More patients had a positive result for IgM aPI (32%) than for IgG aPI (16%) (Table 1). Only two patients had raised levels of both isotypes and five patients elevated levels of one isotype (one IgG, four IgM). The remaining 12 patients were considered negative by our criteria.

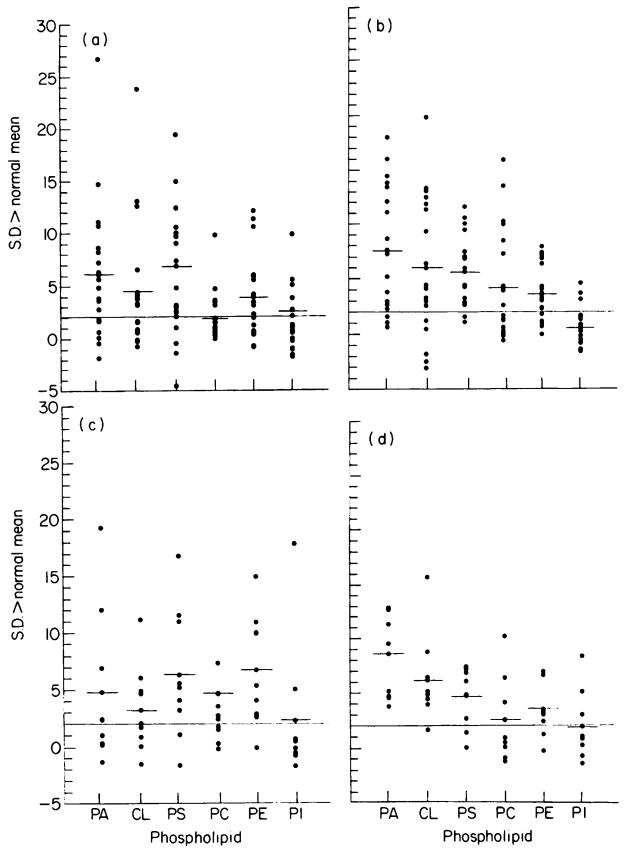

Fig. 1. Anti-phospholipid antibody (aPLA) binding to individual phospholipids showing (a) IgM and (b) IgG levels in 19 patients with uncomplicated *Plasmodium falciparum* infections, and (c) IgM and (d) IgG levels in nine patients with *P. vivax* malaria (Group C). Horizontal bars represent the mean levels for each phospholipid specificity. PA, Phosphatidic acid; CL, cardiolipin; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

Anti-PI in African children with non-severe compared with severe (cerebral) falciparum malaria

Levels of aPI in Groups D (non-severe malaria) and E (severe cerebral malaria) were compared and any differences statistically evaluated. The results, expressed as s.d. above the normal mean, are shown in Fig. 2.

Both IgG and IgM aPI were lower than in adults with primary falciparum infections (Group C). Only one child with cerebral malaria had a raised IgG aPI (Fig. 2a), and IgG levels between the two groups were not significantly different.

For IgM aPI, however, there was a clear statistically significant difference ($P < 0{\cdot}05$) between the two groups. Whereas all children with non-severe malaria had levels elevated above the normal mean (although only two were significantly elevated (> 2 s.d. above the normal mean)) as seen in Fig. 2b, only 3/7 children with cerebral malaria had similarly raised levels, and none was significantly different from normal.

aPLA in SLE controls

Raised IgG aPLA levels were obtained against anionic phospholipids with PA > PC (data not shown). The only significantly raised IgM aPLA was to the anionic mix (mean = 3·07, 1 s.d. = 1·86).

DISCUSSION

The present study shows that both *P. falciparum* and *P. vivax* infections are associated with the appearance of elevated plasma aPL antibodies with a broad spectrum of PL specificity. As in the autoimmune diseases, SLE and aPLS, most malaria patients were positive (values > 2 s.d. above a normal mean) for antibodies to the anionic phospholipids (PS, PC, and PA) with the exception of PI, where levels were found in a much lower percentage of patients (see below). Levels of aPLA in malaria to anionic PL approximated levels obtained from patients with SLE tested concurrently.

Antibodies in malaria samples reacted with a wide range of individual phospholipids in the solid-phase immunoassay, a situation also observed in autoimmune disorders, for example in aPLS where patients' sera were found to cross react with the majority of PL [1]. One of the major components of the aPL

Table 1. Percentage of malaria patients considered positive for IgG and IgM anti-phospholipid antibodies (aPLA) to anionic and cationic phospholipids

	IgG		IgM	
Phospholipid	*P. falciparum* ($n = 19$)	*P. vivax* ($n = 9$)	*P. falciparum* ($n = 19$)	*P. vivax* ($n = 9$)
CL	74 (14)	89 (8)	58 (10)	44 (4)
PA	79 (15)	100 (9)	68 (13)	56 (5)
PS	89 (17)	78 (7)	79 (15)	78 (7)
Anionic mix	84 (16)	67 (6)	84 (16)	56 (5)
PC	47 (9)	45 (4)	26 (5)	56 (5)
PE	68 (13)	78 (7)	68 (13)	89 (8)
Cationic mix	53 (10)	33 (3)	37 (7)	23 (2)
PI	16 (3)	33 (3)	32 (6)	23 (2)

Figures in parentheses are numbers of patients positive for aPLA.

CL, cardiolipin; PA, phosphatidic acid; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

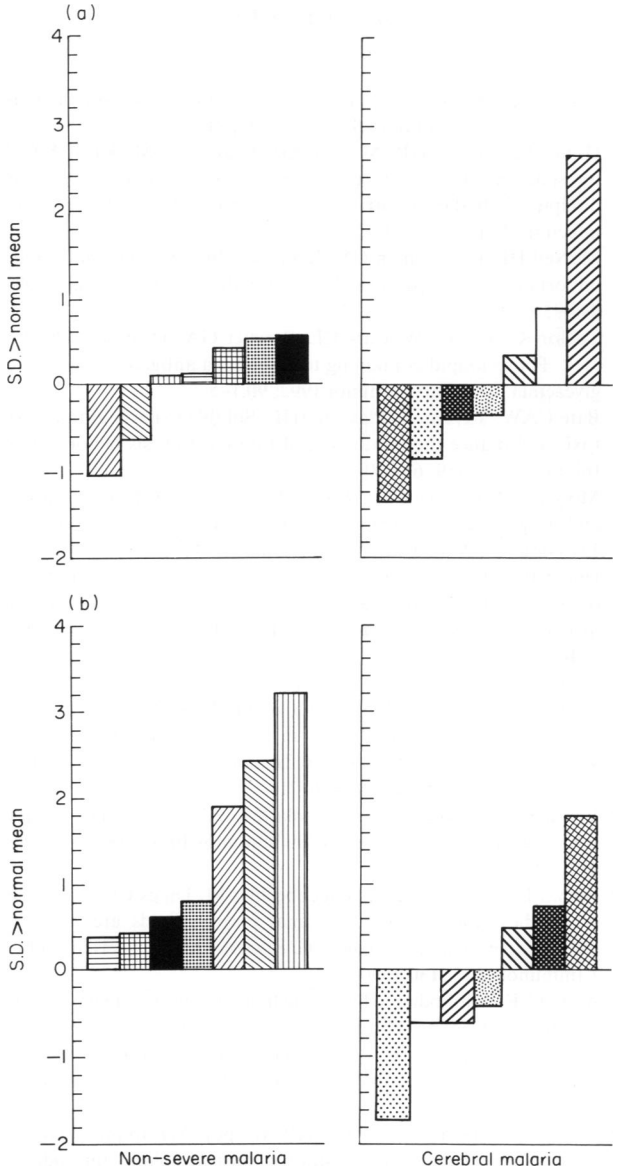

Fig. 2. Anti-phosphatidylinositol (PI) IgG (a) and IgM (b) levels in African negro children with non-severe and severe (cerebral) falciparum malaria (Groups D and E). Each patterned bar represents an individual patient.

response to anionic PL are antibodies with high CL-binding activity, and these have been described to occur in a variety of infections such as AIDS, infectious mononucleosis, syphilis, tuberculosis, hepatitis A [2,9] and also parasitic infections such as African trypanosomiasis [10] and Lyme disease [1].

Antibodies reacting with CL were elevated in over 75% of our malaria patients, with many *P. falciparum* cases having very high (>6 s.d. above the normal mean) IgG levels, considerably higher than levels in some of the SLE sera examined and higher than the SLE group mean.

Malaria patients, like those with SLE, had raised levels of aPL of either one isotype alone, or a mixture of IgG and IgM. There was no correlation between the levels of both isotypes, unlike that described for naturally occurring anti-cholesterol antibodies in human sera [11]. Recent reports on the prevalence and significance of aPL isotypes measured by ELISA, in particular aCL, have been discrepant and conflicting, and probably relate to methodological variables [12]. The current general opinion is that isotype prevalence has no clinical significance, although it might provide some indication of the stimulus for the production of aPLA (see below).

What is the consequence of elevated aPL, and do they have any pathological or beneficial effect in malaria? Information obtained from studies in autoimmune disease indicates that the presence of antibodies with specificity for cardiolipin is deleterious and confers an increased risk of arterial and venous thrombosis and thrombocytopenia. However, these complications are not always found in infections where aCL are raised to a similar extent, implying a qualitative difference between aCL found in these situations. Anti-CL purified from autoimmune sera bound CL only in the presence of β_2-glycoprotein 1 (β_2-GP1) found in fetal calf serum (FCS) which is added to the ELISA diluent. Anti-CL from patients with malaria and other infections did not require this protein for binding [2]. The authors related this feature to the presence or absence of thrombotic complications respectively, although the mechanism by which antibodies directed to a presumed β_2-GP1/CL complex may predispose to clotting disorders has not been explained. Arterial and venous thrombosis is not a feature of malaria, although a thrombocytopenia of unknown etiology is [13,14].

Anti-CL and aPE antibodies bind to platelets and are thought to be responsible for the thrombocytopenia of SLE and aPLS [15]. Could they also be responsible for the thrombocytopenia in malaria given the elevated levels of aCL and aPE, and, if so, how? It has been suggested that aPLA can bind to PS exposed on the activated platelet membrane, thereby causing membrane damage, increasing platelet removal by the reticuloendothelial system, and shortening platelet survival [1,16]. Activated platelets are found in the circulation of malaria patients, have bound immunoglobulin of undetermined antigen specificity and exhibit a shortened survival [14].

Explanations for the presence of elevated levels of aPLA in malaria are several. They may be part of the polyclonal B lymphocyte activation and expansion of B cell clones with autoantibody production that are features of malaria [17,18]. However, this does not explain why all malaria patients in our study did not have raised levels, and why the titre of certain aPLA, such as aPS and aPE, are markedly elevated whereas others such as aPI are uncommon or at low level.

An alternative explanation could be that parasite-derived products or malaria-modified (or cryptic) host antigens have specific PL components which are antigenic. Loss of the normal transbilayer phospholipid asymmetry in parasitized erythrocytes results in the appearance of PS and PE and decreasing PC in the outer leaflet [19]. The exposure of PS on the outside of the cell may be the stimulus for the production of aPS. Furthermore, opsonization of parasitized cells by aPS would enhance their phagocytosis.

An important observation in this study was the raised levels of IgG and IgM specific for PI, which have not been described previously in infections. Although most aPLA bind to an epitope which includes a phosphate ester head common to other phospholipids, aPI antibodies recognize PI specifically.

Phospholipids, in particular PI, are essential components of cell membranes that can serve as a source for intracellular

signalling molecules. Recently a glycolipid 'exoantigen' released from the membrane of *P. falciparum* merozoites has been described which is glycosylphosphatidylinositol (GPI) containing myristic and palmitic acids [20,21]. This GPI, free or protein-associated, induced TNF and IL-1 production by mouse macrophages, and regulated glucose metabolism by adipocytes *in vitro*. Antibody raised to malaria GPI inhibited these effects. Similarly, others have confirmed that the active component of malaria exoantigens or 'toxins' is phosphate bound to inositol, and antisera raised against PI effectively inhibited TNF secretion by macrophages stimulated by *P. falciparum* and *P. vivax* exoantigens *in vitro* [22]. Since the raised plasma TNF found in malaria has been linked to disease severity, with exceptionally high levels recorded in cerebral malaria [23], there may be a rationale for the development of an antiglycolipid (anti-disease) vaccine [24] which would prevent TNF elevation and the attendant hypoglycaemia.

It is conceivable that the raised aPI described here functions in a similar capacity. Antibodies to PI would be produced to the PI component of exoantigens released into the blood circulation during episodes of schizogony. If antibodies to PI are protective, then individuals with severe (cerebral) malaria might be expected to have lower aPI levels (and higher TNF) than patients with uncomplicated disease. Indeed this was the case in the present study, albeit only within a small group of individuals, where the African children with cerebral malaria had statistically significantly lower IgM aPI than did age-matched children with uncomplicated disease. This is an interesting observation which now warrants confirmation with a larger number of individuals.

Finally, a frequently asymptomatic positive direct antiglobulin test (DAT), with immunoglobulin and C3d deposition, is encountered in most patients with SLE and aPLS in association with a high (> 6 s.d. above the normal mean) serum IgG or IgM aCL activity. Eluates made from DAT-positive erythrocytes contained antibody specific for PC and PE [8]. A positive DAT is found in approximately 50% of Gambian children with clinical or asymptomatic falciparum infections, with unparasitized erythrocytes sensitized with IgG and C3d [25]. Eluates made from DAT-positive erythrocytes reacted with merozoites [26]. Whether or not the merozoite-specific antibody on the erythrocytes is directed to a phospholipid determinant which cross-reacts with unparasitized erythrocytes, or represents attachment of malaria phospholipid antigen–antibody complexes, remains to be determined.

We are currently pursuing further studies of aPLA in a larger group of patients with and without cerebral malaria and with varying degrees of immunity and clinical symptoms in an attempt to characterize the specificity, production and function of these antibodies more fully. Our findings here raise the question as to whether: (i) serum-derived aPLA are effective in inhibiting exoantigen-induced TNF production by macrophages; (ii) they play a significant role in removal of parasitized (and non-parasitized) erythrocytes; and (iii) they are responsible for thrombocytopenia and endothelial injury observed *in vivo*.

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