A prognostic role for anti-phosphatidyl choline antibodies in human cerebral malaria

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SUMMARY

Anti-phosphatidyl choline antibodies (αPC) have been measured in adult patients from Orissa, India with *Plasmodium falciparum* infection of varying clinical severity. Significantly raised levels of αPC were observed in infected individuals in comparison with controls. The IgG αPC were found to be generally more than IgM αPC in most cases. The IgG αPC levels were significantly more in those cases of cerebral malaria who recovered fully after quinine administration in comparison with fatal cases not responding to quinine therapy, indicating a role for αPC in prognosis of adult cerebral malaria. There was no significant difference in levels of αPC IgG between non-cerebral and fatal cerebral malaria patients, indicating an absence of a direct protective role in the development of cerebral manifestations. Subgroup typing of IgG with αPC activity indicated IgG3 to be the predominant type, followed by IgG2, IgG1 and IgG4. A significant inverse relationship between serum tumour necrosis factor-alpha (TNF-α) levels and IgG1 antibodies with αPC activity was found, emphasizing the importance of αPC in modifying disease severity. These observations appear to give credence to recent reports in the literature indicating that toxic malarial antigens consist of phospholipids and that antibodies to phospholipids (αPL) inhibit such antigens in experimental systems.

Keywords cerebral malaria   phosphatidyl choline antibodies   *Plasmodium falciparum* prognosis   tumour necrosis factor-alpha

INTRODUCTION

Anti-phospholipid antibodies (αPL) have been extensively studied in systemic lupus erythematosus and many other auto-immune disorders. The role played by αPL in the pathogenesis of diseases is still largely conjectural [1]. Raised levels of αPL have been observed in a variety of infectious diseases such as malaria, syphilis, trypanosomiasis, and leprosy [2,3]. Qualitatively the αPL in autoimmune diseases appear to be different from those observed in such infections [2]. Interest in the role of αPL has been renewed recently in malaria, since the active component of a heat-stable endotoxin-like malarial exo-antigen, capable of inducing tumour necrosis factor-alpha (TNF-α), has been identified to be a phospholipid [4]. With evidence for the role of TNF-α in malarial pathology accumulating progressively [5,6], detailed investigations on αPL in malaria, particularly in patients with cerebral complications, have become crucially important. The observation that antibodies produced in experimental animals to phospholipid could effectively inhibit the release of malarial antigen-induced TNF-α further emphasizes a vital biological role for αPL in malaria [7].

Recent studies performed with sera samples collected from malarial patients in Africa have demonstrated raised levels of αPL [3,8]. While in one of those reports [8] evidence was provided for a protective role by way of providing an 'anti-disease' immune response, the other report [3] indicated that raised titres of αPL were associated with the severity of disease. In the present investigations we have examined sera collected from patients (adults) infected with *Plasmodium falciparum*, with or without cerebral complications, in Orissa, India. The antiphosphatidyl choline (αPC) levels were found to be raised during acute malarial infection. Interestingly, higher IgG αPC levels were associated with good prognosis in cerebral malaria. Further, we also observed a significant inverse relationship between levels of IgG1 αPC and serum TNF-α.

PATIENTS AND METHODS

Patients and sera

Patients (adults) reporting at the out-patient department and/ or admitted to the Department of Internal Medicine at S.C.B.

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Medical College (Cuttack, India), with short history of fever associated with unarousable coma were assessed clinically and Giemsa-stained blood smears examined microscopically for the presence of malarial parasite, and those found positive for *P. falciparum* infection were selected as cases of cerebral malaria for the study. Other causes of encephalopathy such as trauma, hypoglycaemia or intoxication were excluded. The patients were divided into two groups based on post-treatment outcome as follows: Group A, patients with confirmed cerebral malaria in the age group of 20–50 years who recovered completely after adequate intravenous administration of quinine ($n = 15$); Group B, patients with confirmed cerebral malaria in the age group 20–50 years who succumbed to infection despite chemotherapy with quinine ($n = 6$).

Two more groups were taken as controls: Group C, patients in the age group 20–50 years with confirmed *P. falciparum* infection who reported at the out-patient department and were taken as cases of uncomplicated malaria ($n = 8$); Group D, 20 normal, healthy individuals in the age range 16–30 years living in *P. falciparum* endemic areas ($n = 20$).

About 5 ml of blood were collected from each of the patients/controls after informed consent before initiation of quinine (Groups A and B) or chloroquine therapy (Group C). The sera were separated and kept frozen at –20°C until further use.

Lipid ELISA for αPC assay
ELISA was performed utilizing as antigen phosphatidyl choline (PC) (no. P9671) as antigen (Sigma Chemical Co., St Louis, MO) by following a procedure described earlier by Jakobsen *et al.* [3]. Microtitre plates (Titretek, Boston, MA) were coated with 100 μl of 70% ethanol containing 3 μg of the lipid. The solvent was evaporated overnight by incubation at 37°C. The plates were washed in buffer (2 M NaCl, 0·04 M MgSO₄) three times, and blocked for 1 h at room temperature with 2% bovine serum albumin (BSA). After repeated washings 100 μl of human sera diluted 1 : 100 in washing buffer containing 10% fetal calf serum (FCS) were added to the wells in duplicate and incubated at 37°C for 90 min and washings repeated. One hundred microlitres of anti-human IgG (Sigma; no. 3150) or anti-human IgM conjugated with alkaline phosphatase (Sigma; no. 3275) diluted 1000 times were added and incubated at 37°C for 90 min. Enzyme activity was measured by addition of 100 μl of the substrate *p*-nitrophenyl phosphate (1 mg/ml) in carbonate buffer pH 9·4. Absorbance were read in an ELISA reader (BioRad, Richmond, CA) at 405 nm after 2 h incubation at 37°C. To exclude day-to-day variations, results were expressed in ELISA units (EU) using an internal laboratory standard as positive control. The EU were calculated as follows:

$$\frac{\text{OD (sample)} - \text{OD (background)}}{\text{OD (positive control)} - \text{OD (background)}} \times 100$$

The positive control used as laboratory standard for calculation of ELISA units was prepared by making a pool of four malarial sera which showed an absorbance range of 0·50–0·75 for anti-PC IgG and IgM. The same standard pool was used for calculation of ELISA units for both IgG and IgM.

Assay of IgG subclasses with anti-phosphatidylcholine activity
The assay was performed as indicated above for αPC antibody estimation, with some modifications. Sera were diluted 1 : 100

in washing buffer with 10% FCS and 100 μl was added to wells coated with PC, and incubated at 37°C for 90 min. After washing three times, 100 μl of rabbit anti-human IgG1 or IgG2 or IgG3 or IgG4 (Jansen Lab., Brussels, Belgium; nos KH 161-36-A1, KH 161-42-A1, KH 163-01-A2 and 164-02-A3, respectively) diluted 1 : 200 in washing buffer with 10% FCS were added and incubated at 37°C for 90 min. After washing, 100 μl of 1 : 1000 diluted and anti-rabbit IgG conjugated to alkaline phosphatase (Sigma; no. A-8025) were added and incubated at 37°C for 1 h and then at 4°C overnight. The colour was developed by adding 100 μl of *p*-nitrophenyl phosphate (1 mg/ml in carbonate buffer pH 9·4) and absorbance read at 405 nm as described above.

Assay for TNF-α
TNF-α assay was done using an immunoenzymatic assay kit (Medgenix Corp., Ltd., Brussels, Belgium). Briefly, microtitre plates coated with anti-TNF-α were filled with 200 μl of standard TNF-α (15, 50, 150, 1500 pg/ml, respectively), control or sera of the patient, followed by 50 μl of incubation buffer and incubated for 2 h at room temperature. After washing three times, 100 μl of standard (0 pg/ml of TNF) solution and 50 μl of anti-TNF-α–horseradish peroxidase (HRP) conjugate were dispensed into all wells and incubated for 2 h at room temperature. Colour was developed with chromogen tetramethyl benzidine (TMB) and results were read at 492 nm in an ELISA reader. Final values of TNF-α were computed from a standard graph and expressed as pg/ml.

Statistical analysis
Statistical analysis of the results was done using Student's *t*-test, and regression analysis for computing correlations between appropriate parameters.

RESULTS

Distribution of αPC levels in patients with malaria
In comparison with the controls (Group D), patients with malaria (Groups A, B and C) had increased levels of antibodies to phosphatidyl choline (Fig. 1). Both IgG and IgM αPC levels were significantly more ($P < 0·01$) in patients, the increase of IgG being more prominent than IgM αPC. The IgG αPC titres were significantly more ($P < 0·02$) in Group A patients (survivors) in comparison with Group B (non-survivors) and C (non-complicated malaria), as shown in Fig. 1. αPC IgM levels, on the other hand, were not significantly more in survivors (Group A) in comparison with those who did not recover (Group B). These findings indicate a relationship between αPC and prognosis of human cerebral malaria.

IgG subgroups with αPC activity
Since IgG αPC levels were very significantly elevated in all three groups of malarial patients, we attempted IgG subgrouping with αPC activity. Initially a panel of nine patients' sera were tested and IgG4 was found to be undetectable by lipid ELISA. The relative levels of IgG1, IgG2 and IgG3 were measured in individual sera. Table 1 shows the distribution of αPC levels in IgG subgroups. IgG3 was the predominant subclass with αPC activity, followed by IgG2 and IgG1 in all groups (A, B and C) of patients. There was no significant difference ($P > 0·05$) in the

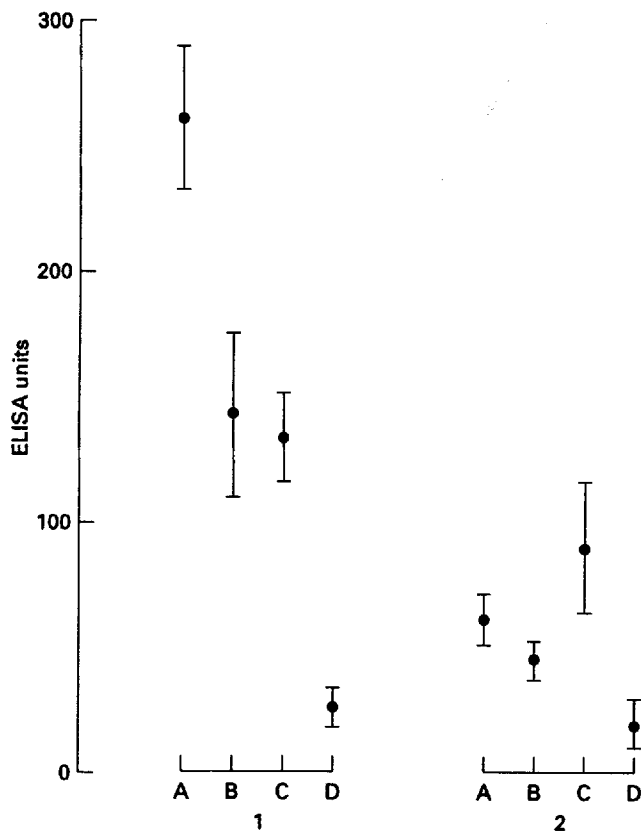**Fig. 1.** Lipid ELISA (mean ± s.d.) of anti-phosphatidyl choline (αPC) antibodies in *Plasmodium falciparum* malaria; IgG (1) as well as IgM (2) αPC are shown in four groups of patients, as described in Patients and Methods; A, cerebral malaria survivors; B, cerebral malaria non-survivors; C, non-complicated *P. falciparum* malaria; D, normal controls.

levels of IgG subgroups with αPC activity between non-complicated and cerebral malaria. However, IgG1 αPC was significantly more (*P* < 0·01) in Group A (survivors) compared with Group B (non-survivors). The levels of IgG2 and IgG3 αPC levels between Groups A and B were, however, comparable.

Relationships between IgG αPC antibodies and TNF-α

Since phospholipid antibodies have been shown in experimental systems to inhibit TNF-α release by malarial exo-antigen,

Table 1. Lipid ELISA-IgG subclass typing of antiphosphatidylcholine (αPC) antibodies in *Plasmodium falciparum* malaria†

Group	IgG1	IgG2	IgG3
A, n = 15	0·21 ± 0·11*	0·30 ± 0·13**	0·61 ± 0·38**
B, n = 6	0·10 ± 0·08	0·27 ± 0·16	0·46 ± 0·24
C, n = 8	0·19 ± 0·05	0·32 ± 0·15	0·53 ± 0·25

*P < 0·01 for Group A *versus* Group B for IgG1; **P > 0·05 for IgG2 and IgG3.

†Absorbance at 405 nm.

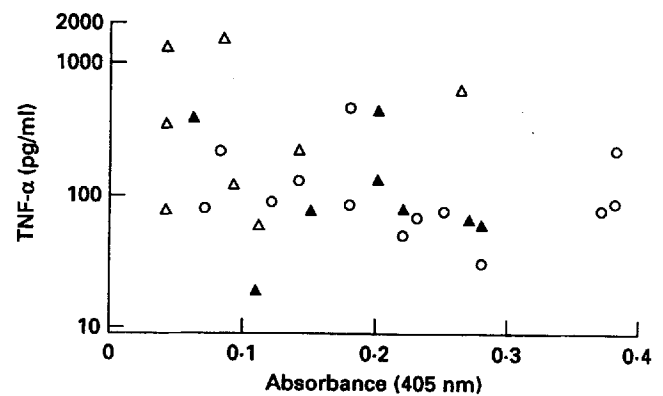**Fig. 2.** Inverse relationship between circulating tumour necrosis factor-alpha (TNF-α) and IgG1 with anti-phosphatidyl choline (αPC) activity in *Plasmodium falciparum* malaria; O, cerebral malaria survivors; △, cerebral malaria non-survivors; ▲, non-complicated *P. falciparum* malaria. (*r* = −0·365, *P* < 0·05.)

we looked for a correlation, if any, between αPC and circulating TNF-α in malaria. A statistically insignificant inverse correlation was observed between TNF-α and IgG αPC levels (*r* = −0·348, *P* > 0·05; Fig. 2). However, a significant correlation was seen when IgG1 αPC levels were compared with TNF-α (*r* = −0·365, *P* < 0·05). Such a significant relationship was not observed when IgG2 or IgG3 αPC were compared with TNF-α levels (*r* = −0·186 and *r* = −0·106 for IgG2 and IgG3, respectively).

DISCUSSION

The present study indicates a significant rise in αPC in human *P. falciparum* infections. While these basic findings in general confirm earlier observations of increased phospholipid antibodies reported from Africa in *P. falciparum* malaria [3,8], the results are qualitatively different. For example, we found a substantial increase in IgG αPC in adults with cerebral malaria, while in the earlier reports IgM was the predominant antibody with anti-phospholipid activity in African children [3]. Since IgG was the predominant isotype with αPC activity, we analysed IgG subclasses and found the distribution in the following order: G3 > G2 > G1 > G4. Quantitatively the distribution of IgG subclass in normal sera is G1 > G2 > G3 > G4. We are not aware of any published work on IgG subgrouping with αPC activity in human cerebral malaria.

Jakobsen *et al.* [3] correlated the rise in IgM αPC to severity of the disease, while Facer & Agiostratidou [8] produced evidence for a protective role of IgM anti-phosphatidylinositol (PI) in children with severe malaria. Separation of patients with cerebral malaria into two categories based on prognosis of the disease in this study has allowed us to evaluate the role of αPC in adult cerebral malaria. If the antibody levels in Group A (cerebral malaria survivors) are compared with group C (non-complicated *P. falciparum* malaria), the increase in IgG αPC appears to correlate with severity of the disease, but comparison between group A (cerebral malaria survivors) with group B (cerebral malaria non-survivors), makes the increase in IgG αPC indicative of a protective role. The biological role of phospholipid antibodies in infectious diseases has been the subject of debate, particularly in malaria. Preliminary evidence

provided by previous workers [3,8] tends to indicate a dual role, namely in severity as well as protection of the disease. However, a closer and critical examination of anti-PC in cerebral malaria and correlation with prognosis, as was done in this study, indicate a possible protective role for αPC in cerebral malaria.

A significant role for TNF-α levels in prognosis has been documented in African children with cerebral malaria [5,6]. We have attempted to correlate TNF-α levels with αPC in our patients, since experimental studies have indicated that phospholipid antibodies inhibit malarial exo-antigen-induced TNF-α release both *in vitro* and *in vivo* [9].

There was an inverse correlation between IgG αPC levels and serum TNF-α, although it was insignificant. However, when individually the IgG subclasses were analysed, there was a significant inverse correlation between αPC IgG1 and TNF-α. Such an association was not observed for the other IgG subclasses, or with IgM αPC. Furthermore, of all the IgG subclasses with anti-PC activity, only IgG1 levels were significantly more in Group A (cerebral malaria survivors) in comparison with Group B (cerebral malaria non-survivors).

Evidence for a definitive protective role for phospholipid antibodies in anti-disease immunity in human malaria in our opinion is yet to come. Recently Bate & Kwiatkowski [10] have produced very preliminary evidence in this direction. Purified IgM and not IgG from a single patient with *P. falciparum* infection was demonstrated *in vitro* to inhibit malarial exo-antigen-induced TNF-α release. We are currently purifying IgG from patients with raised as well as low levels of αPC to perform *in vivo* experiments using toxic *P. berghei* or *P. falciparum* exo-antigen in mice primed with D-galactosamine. These and similar experiments with affinity-purified human αPL will hopefully establish the protective role if any for phospholipid antibodies in anti-disease immunity in malaria.

ACKNOWLEDGMENTS

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