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Immunization of mice with phosphatidylcholine drastically reduces the parasitaemia of subsequent *Plasmodium chabaudi chabaudi* blood-stage infections

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SUMMARY

It has been suggested that phospholipids and antibodies directed against phospholipids are important in the pathology of malaria. We have investigated the influence of immunizations with phospholipids on the course of subsequent blood-stage *Plasmodium chabaudi chabaudi* infections in ICR inbred mice. We observed a significant reduction in the parasitaemia following immunization with phosphatidylcholine (PC), but not with phosphatidylethanolamine (PE) immunization. At the peak of the infection, PC-immunized mice displayed a T-helper 2 (Th2)-type cytokine production pattern, whereas PE-immunized or non-treated controls displayed a cytokine production pattern of the T-helper 1 (Th1) type. Serum immunoglobulin transfer from PC-immunized mice protected naive mice in a similar fashion to PC-immunization, demonstrating that the observed reduction of parasitaemia was caused by the presence of PC-specific antibodies.

INTRODUCTION

Plasmodia extracts, termed malaria toxins, are involved in the pathogenesis of murine malaria.[1–6] The active fraction of these extracts is heat stable and protease resistant. However, its activity can be destroyed by digestion with lipases or by mild base hydrolysis, indicating that the active compounds might be phospholipids, or phospholipid linked.[7,8] Among its properties, this fraction induces tumour necrosis factor-α (TNF-α) production[9] causing various changes in vascular endothelium.[10] Malaria toxins also induce hypoglycaemia, probably by acting directly on the signal transduction cascade downstream from the insulin receptor.[1,11]

It has been demonstrated that the phospholipid composition of the erythrocyte membrane changes upon *Plasmodium* infection[12] and that phosphatidylcholine (PC) is the major membrane phospholipid in *Plasmodia*.[13] Moreover, studies in humans demonstrated that high antibody titres against PC[14–16] and other phospholipids are present in sera of patients with malaria.

By immunizing mice with phospholipids coupled to merozoite surface protein (MSP)[2] or to keyhole limpet haemocyanin (KLH),[9] it has been possible to obtain monoclonal antibodies that are able to neutralize the TNF-inducing activity of malaria toxins *in vitro*.

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Abbreviations: IRBC, infected red blood cells; PC, phosphatidylcholine; Pcc: *Plasmodium chabaudi chabaudi*; PE, phosphatidylethanolamine.

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In the present study, we investigated the influence of immunization with PC on the course of subsequent *Plasmodium chabaudi chabaudi* (Pcc) infections in ICR mice. Non-immunized animals and mice immunized with phosphatidylethanolamine (PE) were used as controls.

MATERIALS AND METHODS

Animals

Female ICR (ICR/Thlbmp) mice, 8–10 weeks old, were purchased from BRL (Füllinsdorf, Switzerland). Mice were maintained on standard mouse chow and water *ad libitum*.

Parasites and infection

Plasmodium chabaudi chabaudi AS was a kind gift from Dr D. Walliker (Edinburgh, UK). Infections were performed by intraperitoneal (i.p.) injection of 10^6 parasitized red blood cells (PRBC). Parasitaemia was scored on Giemsa-stained tail-blood smears.

Coupling of phospholipids to purified protein derivative

L-α phosphatidylcholine (L-α egg yolk lecithin; PC), and L-α phosphatidylethanolamine (L-α egg yolk kephalin; PE) were purchased from Sigma (Buchs, Switzerland). Purified protein derivative from tuberculin (PPD) was a kind gift from Dr Lang, Swiss Serum and Vaccine Institute, Bern, Switzerland.

PC and PE were coupled to PPD at a ratio of 1·6/1. Briefly, 5 mg phospholipid were dissolved in 100 µl of ethanol and then diluted to 5 mg/ml with 0·25 M phosphate buffer, pH 7·2, by stirring on ice. Phospholipids, 1·5 mg in 300 µl of phosphate buffer, were added to 0·6 mg PPD dissolved in 300 µl of phosphate buffer. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimid-HCl (5 mg; Sigma) dissolved in 50 µl of H_2O was added by stirring at 4° for 20 min. The remaining active sites

were blocked with 100 µl of 1 M glycine. Coupled compounds were dialysed against phosphate-buffered saline (PBS), adjusted to 3 ml with PBS and stored at 4°.

Immunization

Mice were injected three times i.p. with 300 µl of PC-PPD or PE-PPD suspension, containing ≈50 µg phospholipid. The animals were boosted 3 weeks after the first injection, and by a third injection 2 weeks later. Bleeding, spleen excision, and/or infection were performed 10 days after the last injection.

Anti-PC and anti-PE enzyme-linked immunosorbent assay (ELISA)

Maxisorp microtitre plates (Nunc, Life Technologies, Basel, Switzerland) were coated with 100 µl of a solution of 30 µg/ml PC or PE in 70% ethanol. The solvent was evaporated overnight by leaving the plates without lids under the airstream of a sterile hood. The plates were washed three times in water and blocked for 60 min at room temperature with PBS + 1% bovine serum albumin (BSA) and 0·1% Tween-20 (PBS-BSA-T). Serum (100 µl/well) diluted in PBS-BSA-T was added, and incubated at 37° for 90 min. After washing, 100 µl of anti-mouse immunoglobulin M (IgM), immunoglobulin G (IgG)1, IgG2a, IgG2b or IgG3-specific alkaline phosphatase (ALPH)-labelled goat antibodies (Southern Biotechnology Associates Inc., Bioreba, Reinach Switzerland), diluted in PBS-BSA-T was added, and incubated for 60 min at 37°. After washing, 100 µl/well *p*-nitrophenylphosphate was added as substrate and the colour reaction was recorded at 405 nm. Effective absorbance (*A*) values were calculated as follows: [(*A* of test sample) – (*A* blank in coated plate)] – [(*A* of test sample in non-coated plate) – (*A* blank from non-coated plate)]. For all the immunoglobulin subtypes, antibodies against the respective phospholipids were detectable up to the 1:800 dilution of the sera of the immunized mice. No antibody directed either against PC or PE was detected in the sera from naive mice, where the *A* values remained at background levels for all dilutions.

Preparation of Pcc antigen

ICR mice were infected by i.p. injection of Pcc-parasitized erythrocytes, as described above. After development of a parasitaemia above 60%, the animals were killed and bled by cardiac puncture. Blood cells were washed twice with HBSS + 0·5% EDTA (Hanks' balanced salt solution without Ca^{2+} & $Mg;^{2+}$ Life Technologies). Red blood cells parasitized with 99% ring-forms were obtained by centrifugation over a discontinuous Percoll gradient (55–65–75%), and resuspended to 5×10^9/ml in HBSS. The suspension was sonicated on ice with 28 kilocycles, for 4 cycles of 30 seconds each, filtered through a 0·22 micron membrane, aliquoted and stored at −70° until use.

As antigen for cultures, Pcc aliquots were diluted 5× in IMDM (Iscove's modified Dulbecco's medium with L-glutamine, supplemented with 3·024 g/l $NaHCO_3$, 5×10^{-5} M 2-mercaptoethanol, 10^5 U/l penicillin–streptomycin mixture, 10 ml/l of a ×100 concentrated minimal essential medium (MEM) non-essential amino acids mixture; all reagents from Life Technologies), and added in a volume of 10%, which represented approximately the amount of antigen from $1{\cdot}98 \times 10^8$ PRBC/culture.

Pcc-specific antibody ELISA

Pcc-specific antibodies were measured by ELISA as described previously.[17] Briefly, plates were coated with 100 µl/well Pcc trophozoite antigen and blocked with PBS + 1% BSA (PBS-BSA). Sera were diluted in PBS-BSA and incubated in the plates. ALPH anti-mouse immunoglobulin isotype antibodies (Southern Biotechnology Associates Inc.) were added, followed by *p*-nitrophenylphosphate (*p*-NPP; Bio-Rad, Glattbrugg, Switzerland) in carbonate buffer. The *A*405 was measured using an ELISA reader and effective *A* values were calculated as described above.

ELISAs for TNF-α, interferon-γ (IFN-γ), interleukin-4 (IL-4) and interleukin-5 (IL-5)

ELISAs were performed as described previously.[18] Briefly, plates were coated with rat monoclonal antibody (mAb) anti-mouse TNF-α (clone MP6-XT22; Endogen, Bioreba, Reinach, Switzerland), rat mAb anti-mouse IFN-γ (clone R4-6A2), rat mAb anti-mouse IL-4 (clone BVD4-1D11) or rat mAb anti-mouse IL-5 (clone TRFK-5; the latter three were Minikits from Endogen). The plates were blocked with PBS + 1% BSA and incubated overnight after addition of serum samples and standards. Affinity-purified rabbit IgG anti-mouse TNF-α (Endogen) or biotinylated anti-mouse IFN-γ, IL-4, or IL-5 (Endogen) were used as secondary antibodies. ALPH-labelled goat anti-rabbit $F(ab')_2$ fragment (Jackson Immuno-Research, Milian, La Roche, Switzerland) for TNF-α or horseradish peroxidase (HRPO)-conjugated streptavidin (Zymed, Bioreba, Reinach, Switzerland) for IFN-γ, IL-4 and IL-5 were used for detection. *p*-nitrophenylphosphate (p-NPP, Bio-Rad) for TNF-α or 1,1′-trimethylene-*bis* (4-formylpyridinium bromide) (TMB; KPL, Bioreba, Reinach, Switzerland) for IFN-γ, IL-4 and IL-5 were added and colour reactions were recorded at 405 nm for ALPH and 620 nm for HRPO.

Single spleen cell suspension and short-term cultures for enumeration of spot-forming cells (ELISPOT) assays

Spleen excision from mice was performed after killing by cervical dislocation. Single-cell suspensions were obtained by gently squeezing the organ through a sterile steel mesh. After washing with HBSS, cell viability was determined by ethidium bromide/acridine orange dye exclusion and the cells were resuspended to 5×10^6 viable cells/ml in IMDM containing 10% FBS (fetal bovine serum). The suspension was distributed (1 ml/well) in 24-well culture plates (Costar, Integra, Wallisellen, Switzerland). After addition of 100 µl of 5× diluted Pcc antigen, the plates were incubated at 37° in a 5% CO_2 water-saturated atmosphere for 18 hr.

TNF-α, IFN-γ, IL-4 and IL-5 ELISPOTs

ELISPOTs were performed as described previously.[18] Briefly, plates were coated with rat mAb anti-mouse TNF-α (clone XT3, Endogen), rat mAb anti-mouse IFN-γ (clone R4-6A2, Endogen), rat mAb anti-mouse IL-4 (clone 1D11, Endogen) or rat mAb anti-mouse IL-5 (clone TRFK4, Endogen). Plates were blocked with PBS + 1% BSA and serial dilutions of spleen cells from short-term cultures were added. After incubation, cells were removed and rabbit IgG anti-mouse TNF-α (Endogen), biotinylated rat anti-mouse IFN-γ (clone XMG1·2, Endogen), biotinylated anti-mouse IL-4 (clone 24G2,

Endogen) or biotinylated anti-mouse IL-5 (clone TRFK-5, Endogen) was added. ALPH-conjugated goat anti-rabbit $F(ab')_2$ fragment (Jackson Immuno-Research, Milian, La Roche, Switzerland) for TNF-α, or ALPH-conjugated avidin (Sigma, Buchs, Switzerland) for IFN-γ, IL-4 and IL-5 were added as conjugates. After incubation, 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Bio-Rad) was added as substrate. The colour reaction was stopped by rinsing the plates with distilled water and spots were counted under 16× magnification.

Preparation of serum immunoglobulin and transfer experiments

Ten days after the last phospholipid (PC and PE) injection, five animals were bled by cardiac puncture and their sera were pooled and diluted with PBS.[15] After elution with 3 M potassium thiocyanate from a goat anti-mouse IgG (whole molecule)-agarose column (Sigma), antibodies were concentrated with polyethylene glycol 35 000 and dialysed against PBS. Measured by Bio-Rad assay, the protein concentration was 5·9 mg/ml for PC and 5·1 mg/ml for PE. Serum immunoglobulin solutions were adjusted to 5 mg/ml with sterile PBS and stored at −70° until use. For PC and PE, respectively, the composition of the transferred immunoglobulin pools, measured by ELISA and quantified by comparison to mouse myeloma standards, were as follows: IgM, 7·9% and 4·7%; IgG1, 17·7% and 28·8%; IgG2a, 9·3% and 6·6%; IgG2b, 6·1% and 6·7%; IgG3, 13·5% and 18·6%; IgA, 1·8% and 2·8%; immunoglobulin E (IgE), not found.

Mice were injected intravenously with 0·3 ml of immunoglobulin solution containing 1·5 mg protein, 24 hr before infection with Pcc.

Statistical analysis

All experiments were repeated at least twice. Results were analysed for statistical significance by using the Student's *t*-test. A $P \leq 0{\cdot}005$ was considered as significant.

RESULTS

The effects of immunization with PC on the course of subsequent Pcc infections were analysed. Controls were either non-immunized (NI) mice or mice immunized with PE, which had been shown in pre-experiments not to influence the course of Pcc infections.

Specific antibody production upon immunization with phospholipids

The choice of pure PPD as carrier was based on the fact that this protein promotes primarily a cellular response, and because its use allowed us to immunize animals without lipidic adjuvants, which might have induced artefacts.

Figure 1 summarizes the profiles of PC- and PE-specific antibody titres in sera from 10 mice per group, 10 days after the third immunization. For both PC and PE, specific IgM and IgG subtype titres were comparable. Antibodies directed against PC or PE were not detectable in naive control mice (data not shown). Moreover, no antibody directed against PE was detected by ELISA in the sera of PC immunized mice, and vice versa. This indicates that there was no cross-reaction between antibodies directed against PE or PC.

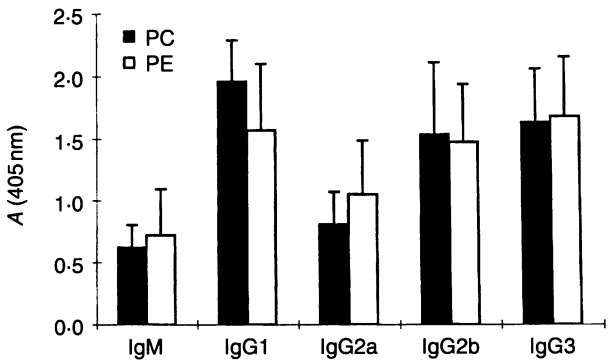

Figure 1. Phospholipid-specific antibody production after immunization with PC or PE. Mice ($n=10$) were immunized three times with PPD-coupled PC or PE. PC and PE specific antibody production was measured 10 days after the last immunization. Results are presented as mean effective *A* (see the Materials and methods) ± SD measured for 1:50 dilutions, where all samples were in the linear range of the ELISA. In control mice sera, only background levels of PC or PE-specific antibodies were measured. PC and PE-specific antibodies did not cross-react.

Course of Pcc infections after immunization with phospholipids

Antibodies to phospholipids can inhibit the stimulation of TNF-α production by malaria toxins.[2,9] We therefore investigated whether the production of such antibodies would influence the course of a blood-stage Pcc malaria infection in mice.

As illustrated in Fig. 2(a), we found a significant reduction of the parasitaemia peak and a faster clearance of parasites in mice immunized with PC. Control immunization with PE did not alter either the height of the parasitaemia peak or the clearance rate of the parasite, as compared with NI mice.

The cytokine production at the time of the parasitaemia peak

Table 1. Immune response pattern at the parasitaemia peak of *Plasmodium chabaudi chabaudi* infections. The immune response pattern was assessed either by measuring the concentrations of cytokines in sera by ELISA, and by enumerating the number of spot-forming cells in 10^6 spleen cells by ELISPOT

Immunization		TNF-α	IFN-γ	IL-4	IL-5
PC	SFC†/10^6	19±11*	53±13*	161±19*	209±33*
	pg[b]‡/ml	13±9*	88±9	182±13*	242±19*
PE	SFC/10^6	127±37	182±24	46±11	41±9
	pg/ml	82±21	123±15	51±8	61±15
NI	SFC/10^6	165±22	204±12	71±10	53±9
	pg/ml	103±11	144±12	48±13	55±4

†Spot-forming cells (SFC), measured by ELISPOT. Mean±SD/10^6 spleen cells of duplicates from 10 animals.

[b]Mean±SD, measured by ELISA, of triplicate serum samples from 10 animals.

*Statistical difference between PC and PE: $P<0{\cdot}005$ Student's *t*-test.

NI, non-immune controls; PC, phosphatidylcholine;PE, phosphatidylethanolamine.

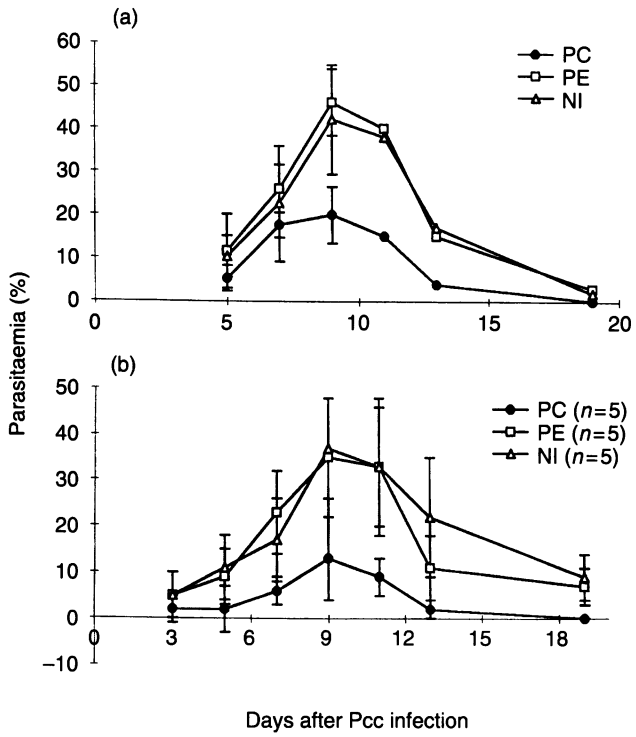

Figure 2. (a) Course of *Plasmodium chabaudi chabaudi* (Pcc) infections in mice ($n=8$) immunized with PC or PE, or not immunized (NI). Results are represented as mean percentage of parasitized erythrocytes ± SD. In this experiment, mice were killed for the analysis of cytokine production at day 9 and only one mouse was followed-up afterwards. (b) Course of Pcc infections in naive mice ($n=5$), after serum immunoglobulin transfer from PC or PE-immunized mice. Results are represented as mean percentage of parasitized erythrocytes ± SD.

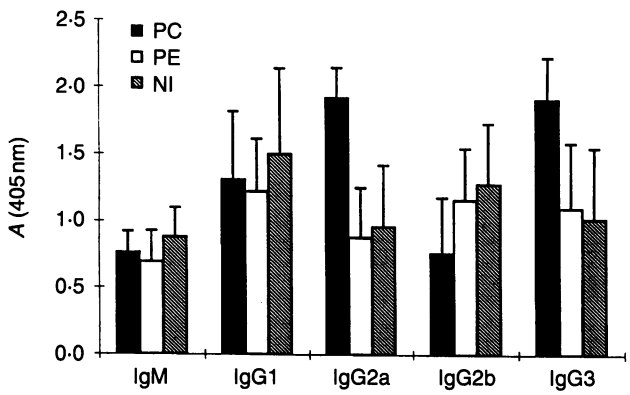

Figure 3. *Plasmodium chabaudi chabaudi* (Pcc)-specific antibody production after immunization with PC or PE. Mice ($n=5$) were immunized three times with PPD-coupled PC or PE, or not immunized (NI), and infected with 10^6 Pcc-infected red blood cells 10 days after the last immunization. Pcc-specific antibodies were measured at day 9 of the infection. Results are presented as effective $A \pm$SD of 1:50 dilutions.

was analysed by ELISPOT and ELISA (Table 1). The mice immunized with PC displayed a clear T helper 2 (Th2) pattern, indicated by elevated serum levels of IL-4 and IL-5, and the presence of a high number of IL-4 and IL-5 spot-forming cells (SFC) in the spleen of these mice, in comparison to TNF-α and IFN-γ production. PE-immunized or NI mice displayed a T helper 1 (Th1) pattern at the time of the parasitaemia peak, in agreement with the literature.[19] This was shown by elevated serum levels of TNF-α and IFN-γ and SFC in the spleen, in comparison to IL-4 and IL-5 production.

In accordance with the pattern of cytokine production, PC-immunized mice produced higher titres of Pcc-specific antibodies, at that time of the infection, versus controls. As illustrated in Fig. 3, Pcc-specific IgG2a levels were significantly increased in the PC group (mean A 1·92 ± 0·23) as compared to PE (mean A 0·88 ± 0·37; $P<0·001$) and NI groups (mean A 0·96 ± 0·46; $P<0·001$). Pcc-specific IgG3 levels measured in the PC group (mean A of 1·91 ± 0·32) were significantly higher than those of both PE (mean A 1·10 ± 0·49; $P<0·001$) and NI groups (mean A 1·02 ± 0·53; $P=0·001$). Pcc-specific IgM, IgG1 and IgG2b subclass levels were not statistically different between the different groups. Only background levels of immunoglobulin A (IgA) and IgE could be determined by ELISA in all three groups (data not shown).

Course of Pcc infections after serum immunoglobulin transfer to naive animals

To investigate whether the differences in the parasitaemia course were caused by the production of PC-specific antibodies, we transferred serum immunoglobulins from PC-immunized mice to naive mice and infected them with 10^6 Pcc infected red blood cells (IRBC). As shown in Fig. 2(b), the parasitaemia of the mice injected with serum immunoglobulin from PC-immunized animals was drastically reduced. This reduction was similar to the one observed in Pcc-infected mice after PC immunization.

DISCUSSION

Our results demonstrate that immunization of ICR mice with PC, but not PE, substantially alters the course of a subsequent Pcc infection. This alteration correlates with a shift of the immune response during the primary peak of the infection from Th1 to Th2. Serum immunoglobulin transfer experiments demonstrated that the observed effects are caused by the presence of antibodies directed against PC.

There are several possible explanations for the effects observed in the course of a malaria infection following an immunization with PC. As PC is the most common phospholipid in the membranes of *Plasmodia*,[13] and as the phospholipid composition of the erythrocyte membrane changes upon infection with *Plasmodia*,[12] an immunization with PC could produce a humoral immune response against this molecule. This immune response could eliminate the parasite from the bloodstream, by inducing the destruction of either infected red blood cells or of merozoites before they are able to invade new erythrocytes. Moreover, such antibodies could hinder the invasion of red blood cells by coating the surface of the merozoites. Interestingly, we also detected antibodies directed against PC in sera from mice infected with Pcc, without previous immunization with PC, shortly after the parasitaemia peak (data not shown).

Malaria toxins have been shown to be phospholipids[7] that may play an important role in the development of different

pathologies, e.g. cerebral malaria or fever, through the induction of TNF-α.[3,4,7,8,10] Thus, an immunization with PC could protect the host from some of the pathologies of malaria infections by inducing the production of antibodies against these toxins. This protection could result either from a direct reduction of the up-regulation of TNF-α production by macrophages, or from a down-regulation of Th1-type cytokines, as indicated by the reduced IFN-γ production. Moreover, a reduction of the up-regulation of TNF-α production, leading to a reduction of a Th1-type immune response, might favour a faster switch from a Th1 to a protective Th2 immune response.[19]

In addition, priming with PC could lead directly to a Th2-type immune response during subsequent malaria infections, thus avoiding the various pathologies thought to result from a strong Th1-type immune response. Moreover, as we found significant differences in the production of antibodies against Pcc for the IgG2a and IgG3 isotypes only, it is possible that an immunization with PC preselects protective antibody subtypes.[20] However, the switch factor for IgG2a and IgG3 is thought to be IFN-γ.[21] This suggests that IFN-γ might have been produced earlier in these mice[17] and that it is no longer detectable in sera of the immunized mice at the time of the parasitaemia peak.

In humans, it has been shown that high serum titres of antibodies directed against phospholipids correlate with uncomplicated malaria.[14–16] However, no study has, for ethical and practical reasons, investigated whether such anti-phospholipid antibodies were present before the malaria infection or episode and thus protected the patient from complications, or whether the production of such antibodies was triggered during the investigation period and could be considered as a marker of another reaction, for instance polyclonal B-cell activation. Moreover, as the protection observed in humans correlates with the presence of IgG-type antibodies against phospholipids[16] in the serum, it is likely that the patients had already been in contact with the recognized antigen. Therefore, it can be assumed that the antibodies were already present at the beginning of the malaria infections investigated during these studies. These observations sustain our results and indicate a possible protective role of anti-phospholipid antibodies during malaria.

Investigations on the presence of anti-phospholipid antibodies in the sera of newborn or very young children living in a hyperendemic area for malaria might bring new understanding about the protective capacity of these antibodies. In preliminary studies, we have found that antibodies directed against PC were indeed present in the sera of young children living in an hyperendemic area (data not shown). Further studies are, however, required to determine whether those antibodies are produced by these children or have been acquired by transfer from the mother.

Immunization with phospholipids as a protection against severe malaria complications or the use of monoclonal antibodies against PC for immunotherapy of severe malaria complications might be possible, as discussed by Schofield *et al.*[2] for monoclonal antibodies against glycosylphosphatidylinositol (GPI). However, as PC are also present in the host, unlike GPI which are specific to the malarial parasite, there might be a risk of triggering the immune system to act in an autoimmune fashion; patients with systemic lupus erythematosus (SLE) produce antibodies directed against the phospholipid cardiolipin. Thus, a more profound analysis of the mechanisms leading to the effects observed in our experiments is needed.

In conclusion, our results indicate new perspectives in the research of therapies against malaria. Moreover, we present here a new experimental model for the study of this parasitic disease and its pathologies.

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