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Immunopathology of thrombocytopenia in experimental malaria

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SUMMARY

An early thrombocytopenia was observed in CBA mice during acute infection with *Plasmodium berghei*. This was associated with an increase in bone marrow megakaryocytes and a reduction of normal syngeneic ^{111}Indium-labelled platelet life span. Malaria-induced thrombocytopenia was thus considered to be the result of increased peripheral platelet destruction rather than central hypoproduction. The occurrence of thrombocytopenia was modulated by T-cell depletion. Indeed, thymectomized, irradiated or anti-CD4 monoclonal antibody-treated mice failed to develop thrombocytopenia, although they were infected to the same extent. Conversely, a significant thrombocytopenia was observed in thymectomized mice reconstituted with CD4$^+$ T cells. During the course of infection, a significant inverse correlation was found between platelet counts and platelet-associated IgG. Normal mice passively transfered with serum from syngeneic malaria-infected mice developed thrombocytopenia. The possibility to raise monoclonal anti-platelet antibodies from *P. berghei*-infected animals further suggested a role for an antibody-mediated platelet destruction during acute murine malaria infection. These results indicate that in murine malaria, thrombocytopenia is mediated by immune mechanisms and that CD4$^+$ T cells might be significantly involved.

INTRODUCTION

Thrombocytopenia is a frequent complication during malarial infection, but the mechanisms involved have not been elucidated. Previous studies in *Plasmodium falciparum*-infected patients indicate that this thrombocytopenia is due to peripheral platelet destruction. That immune mechanisms may be involved is suggested by increased levels of platelet-associated immunoglobulins (PAIgG) (Wilson, Neame & Kelton, 1982; Kelton *et al.*, 1983).

A model of cerebral malaria induced by *Plasmodium berghei* infection in CBA mice was analysed in relation to platelet abnormalities. In this model, there was a significant decrease in circulating platelet counts as early as Day 4 of infection. Thrombocytopenia was then progressive until the death of the animals (86·6% mortality between Day 7 and Day 14, 94 mice tested).

In the present study, we attempted to study platelet changes during malaria infection and to define some of the pathogenic mechanisms of acute malarial thrombocytopenia, with particular attention to the T-cell status of the host.

Abbreviations: CM, cerebral malaria; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; mAb, monoclonal antibody; PAIgG, platelet-associated IgG.

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First, in order to establish the peripheral or central origin of the malaria-associated thrombocytopenia, haematological parameters, including bone marrow megakaryocyte density and platelet survival times, were studied. Second, the effects of various T-cell depletion experiments on the occurrence and the degree of *P. berghei*-induced thrombocytopenia were analysed. Third, the production of serum anti-platelet antibodies was evaluated by *in vitro* binding assays and by *in vivo* passive transfer experiments. The production and the specificity of anti-platelet antibodies was also studied with monoclonal antibodies obtained after derivation of hybridomas from spleen cells of *P. berghei*-infected mice.

MATERIALS AND METHODS

Mice

Female CBA/Ca mice, originally obtained from Bomholtgard (Ry, Denmark) and bred in our animal facilities, were 6–8 weeks old at the time of infection.

Parasite strain

Infection was initiated by the i.p. injection of 10^6 *P. berghei* (ANKA strain)-parasitized red blood cells (pRBC). Stabilates were prepared from Day 7-infected mice as described previously (Jayawardena *et al.*, 1977), resuspended in Alsever's solution containing 10% glycerol and stored in liquid nitrogen until use.

Parasitemia
The percentage of parasitized red blood cells was evaluated by Giemsa-stained smears.

In vivo *depletion of T-cell subsets*
Malaria-infected CBA mice were depleted of their $CD4^+$ and $CD8^+$ T-cell subsets by daily injection of the relevant monoclonal antibodies, following an established method which completely eradicates neurological complications (Grau *et al.*, 1986b). The GK1.5 clone producing a rat anti-L3T4 (CD4) IgG2b monoclonal antibody (mAb) was a generous gift from Dr F. W. Fitch, University of Chicago, IL. (Dialynas *et al.*, 1983). The H.35-17.2 clone (rat anti-Ly 2 (CD8) (IgG2b mAb) (Pierres, Goridis & Goldstein, 1982) was kindly donated by Dr H. R. McDonald, Ludwig Institute, Epalinges. Culture supernatants, used as the source of monoclonal antibodies, were injected i.p. (0·5 ml per mouse) daily for 14 days; thereafter they were injected every other day.

ATxBM mice
Adult-thymectomized, irradiated and bone-marrow reconstituted (ATxBM) CBA mice were prepared according to a previously described method (Miller, 1960). Briefly 6-week-old CBA mice were thymectomized, irradiated (850 rads delivered by a ^{60}Co source) and reconstituted with $2·5 \times 10^6$ anti-Thy-1.2-treated syngeneic bone marrow cells injected (i.v.).

Selective reconstitution of ATxBM mice with T-cell subsets
Lymph node cells were obtained from 10 normal CBA mice. Cell suspensions prepared as described (Grau *et al.*, 1986b) were incubated with anti-CD4 and anti-CD8 cytolytic mAbs (Sarmiento, Glasebrook & Fitch, 1980), mouse anti-rat Ig antibody and complement as described above. 10^7 $CD4^+$ $CD8^-$ or $CD8^+$ $CD4^-$ cells were injected i.v. into ATxBM CBA mice, 2 weeks after the irradiation and bone marrow reconstitution.

Platelet counts
Blood (20 μl) was obtained from the retro-orbital plexus using siliconized microcapillaries and immediately diluted 1:100 in Unopette Kits (Becton-Dickinson and Co., Basel). The diluted blood sample was allowed to settle for 20 min in an improved Neubauer haemocytometer and platelets were counted under phase contrast at 400 × magnification. Mice with platelet counts under $0·650 \times 10^6/mm^3$ were considered thrombocytopenic.

Platelet survival studies
The survival of ^{111}Indium-labelled platelets was evaluated according to the previously described method (Grau *et al.*, 1986a). Briefly, platelets isolated from CBA mice were washed in 0·01 M phosphate-buffered saline (PBS) containing 0·3% bovine serum albumin (BSA) (Sigma Chemicals, Zurich), and labelled with ^{111}Indium oxine (Amersham International, Amersham, Bucks, U.K.: 10 mCi/ng Indium). An average of 5 μCi (10^8 labelled platelets) in 0·2 ml were injected i.v. in each recipient mouse. Blood samples were then obtained sequentially and counted in a Packard 5130 gamma counter.

Platelet-associated IgG (PAIgG)
After red blood cell lysis using Unopette kits, the platelets were harvested by differential centrifugation and washed three times in PBS containing 1% BSA and 0·01 M NaN_3 by centrifugation at 2200*g* for 10 min at room temperature. After the last washing, the platelet pellet was solubilized in borate-buffered saline containing 1% Tween 20 and 2% BSA. The platelet lysates (50×10^6 platelets/ml) were kept at 4° until use. The amount of platelet-associated IgG was determined by an enzyme-linked immunosorbent assay (ELISA): platelet lysates were incubated onto Linbro 96-well microtitre plates coated with affinity-purified goat anti-mouse Fc antibody (Cappel Laboratories, West Chester, PA) according to a procedure derived from that of Izui, Eisenberg & Dixon (1979).

Anti-platelet antibodies
Mouse platelet monolayers were prepared by distribution of 50 μl of washed platelets ($10^4/\mu l$) in each cell of Linbro 96-well microtitre plates. The plates were centrifuged for 10 min at 600*g* and 50 μl of 2% formaldehyde solution in PBS were added to each well and incubated for 1 hr at room temperature. The plates were then washed four times with PBS containing 0·05% Tween 20. All the washing steps were performed by immersion-flicking in order to avoid alteration of the monolayer. Serial dilutions of sera to be tested were incubated overnight at 4°, and after four washings, alkaline-phosphatase labelled goat anti-mouse Ig antibody (Cappel Laboratories) was added for 5 hr at 4°. After five additional washes, substrate (nitrophenylphosphate, Sigma Chemicals) was added and the optical density read at 405 nm on a Flow Titertek spectrophotometer.

Immune complexes
Serum levels of soluble immune complexes were determined by using the C1q binding assay described by Zubler & Lambert (1976).

Production of anti-platlet monoclonal antibodies
Spleen cells from *P. berghei*-infected CBA/ca mice were fused with NS-2 myeloma cells. Screening of proliferating hybrids was performed using the platelet monolayer ELISA described above the monoclonal anti-platelet antibodies were derived.

Passive transfer of serum or monoclonal antibodies from thrombocytopenic mice
Sera were obtained from normal and *P. berghei*-infected, thrombocytopenic CBA mice by retro-orbital plexus bleeding. The sera were pooled, decomplemented (by heating at 56° for 30 min) and centrifuged at 2500*g* for 30 min, then millipore filtered. Prior to injection, sera were diluted 1:2 in sterile PBS 0·01 M, pH 7·2.

RESULTS

Kinetics and haematological aspects of thrombocytopenia during infection with *Plasmodium berghei* ANKA in CBA mice

Upon infection with asexual blood stages of *Plasmodium berghei* ANKA strain, there was a drop in platelet counts as early as Day 4 in most of the mice and thrombocytopenia was observed by Day 6–7 ($0·429 + 0·197 \times 10^6$ platelets/μl, controls: $1·050 \pm 0·112 \times 10^6/\mu l$). Platelet counts progressively fell until Day 7–12, when neurological signs appeared in most mice, followed by their death 12–24 hr after onset. The degree of this thrombocytopenia did not differ significantly in mice which did not develop neurological signs (8/60: 13·3% of infected mice).

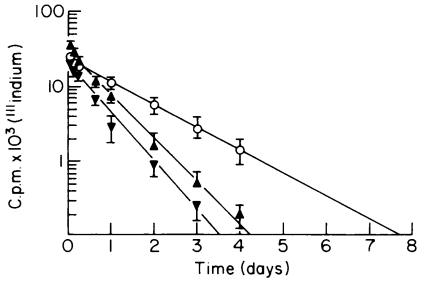

Figure 1. Reduced platelet survival in CBA/Ca mice 7 (▲) and 12 (▼) days after infection with *P. berghei* ANKA; (○) non-infected controls.

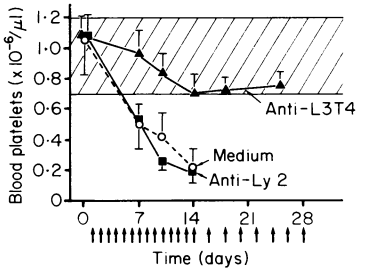

Figure 3. Prevention of thrombocytopenia in *P. berghei*-infected CBA/Ca mice by depletion of the CD4$^+$ T-cell subset. Arrows indicate i.p. injections of GK1.5 (anti-L3T4 = anti-CD4), H.35 (anti-Ly 2 = anti-CD8), or culture medium as control.

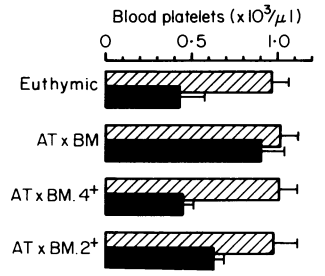

Figure 2. Platelet counts in ATxBM CBA/Ca mice: before (■) and 6 days (■) after infection with *P. berghei*. Absence of thrombocytopenia in *P. berghei*-infected ATxBM CBA/Ca mice. In contrast, ATxBM mice reconstituted with CD4$^+$ T cells develop thrombocytopenia.

Bone marrow specimens were analysed sequentially in *P. berghei*-infected CBA mice. By Day 7, there was a two- to three-fold increase of megakaryocyte (MKC) density (infected mice: 10–12 MKC/ 200 × magnification field; controls: 3–4). Platelet survival studies were performed in *P. berghei*-infected mice. Blood platelets were isolated from normal CBA mice, labelled with ^{111}Indium-Oxine and injected in CBA mice infected with *P. berghei*, 7 and 12 days after infection. As controls, uninfected mice were also injected with ^{111}Indium-labelled platelets. When normal ^{111}In-platelets were injected in malaria-infected mice 7 days after infection with *P. berghei*, reduced platelet survivals were observed (Fig. 1). Platelet survival was also found to be reduced in CBA mice infected for 12 days.

Modulation of malaria-induced thrombocytopenia by manipulation of the T-cell status of the host

Platelet counts were followed up in adult thymectomized, irradiated and bone marrow-reconstituted (ATxBM) CBA mice. By Day 6 of infection, there was no significant thrombocytopenia in ATxBM CBA mice (platelet counts: $0{\cdot}910 \pm 0{\cdot}116 \times 10^6/\mu l$), whereas a significant thrombocytopenia was found in infected euthymic CBA mice (platelet counts: $0.425 \pm 0{\cdot}162 \times 10^6/\mu l$). Although a progressive reduction in platelet counts was observed in ATxBM mice, there was never a significant thrombocytopenia (under $0{\cdot}650 \times 10^6/\mu l$) in these animals. Their death, which occurred by Day 25–30, was associated with severe anemia and overwhelming parasitemia (Fig. 2).

The T-cell dependency of *P. berghei*-induced thrombocytopenia was further studied in experiments using ATxBM selectively reconstituted with CD4$^+$ or CD8$^+$ T-cell subsets. Upon infection with *P. berghei*, it was shown that CD4$^+$ reconstituted ATxBM (ATxBM.4$^+$) mice developed a significantly more profound drop in platelet counts than ATxBM mice (Fig. 2). In ATxBM mice reconstituted with T cells of the CD8$^+$ CD4$^-$ phenotype (ATxBM.2$^+$ mice), there was some degree of thrombocytopenia (Fig. 2).

In vivo depletion of CD4$^+$ T cells prevents the development of malaria-induced thrombocytopenia

The occurrence of thrombocytopenia was evaluated in *P. berghei*-infected CBA mice treated by daily i.p. injections of monoclonal antibody (mAb) GK 1.5, a rat IgG2b specific for the CD4 molecule. Indeed, mAb GK1.5-treated mice were shown to be protected against the development of neurological complications upon infection with *P. berghei* (Grau *et al.*, 1986b). As a control, infected CBA mice were injected with mAb H.35-17.2, a rat anti-CD8 monoclonal antibody of the same isotype. No significant drop in the platelet counts was observed in the GK 1·5 treated mice. In contrast, anti-CD8-treated mice developed a thrombocytopenia to the same extent as untreated infected mice (Fig. 3). Since it was shown that CD4-depleted infected mice failed to produce high levels of TNF (Grau *et al.*, 1987), the role of this cytokine in the generation of thrombocytopenia was evaluated. However, anti-TNF antibody treatment (Grau *et al.*, 1987) of *P. berghei*-infected CBA mice failed to prevent the development of thrombocytopenia ($0{\cdot}450 \pm 0{\cdot}125 \times 10^6$ platelets /μl, $n = 14$).

Immunological parameters of thrombocytopenia associated with *P. berghei* infection

Platelet-associated IgG (PAIgG) were determined during infection with *P. berghei*. In infected mice, there was a significant increase in PAIgG as early as Day 7 ($4{\cdot}6 \pm 3{\cdot}8$ ng IgG/10^6 platelets, controls: $1{\cdot}5 \pm 0{\cdot}7$ ng IgG/10^6 platelets). Between Day 8 ($10{\cdot}0 \pm 7{\cdot}8$) and Day 10 ($17{\cdot}3 \pm 3{\cdot}3$) of infection, PAIgG levels were consistently elevated compared to levels found in normal mice. A significant negative correlation was found between platelet counts and PAIgG (Fig. 4). Sera from *P. berghei*-infected CBA mice were assayed sequentially by using platelet monolayer ELISA for immunoglobulin platelet-binding acti-

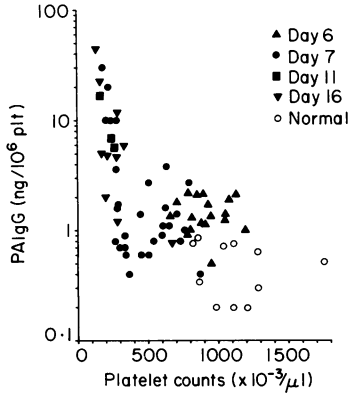

Figure 4. Correlation between PAIgG and blood platelet counts in *P. berghei*-infected CBA/Ca mice.

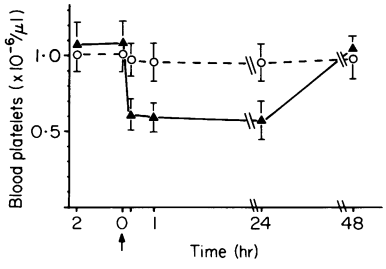

Figure 5. Induction of thrombocytopenia in normal CBA/Ca mice by passive transfer of serum from malaria-infected, thrombocytopenic mice (▲). I.v. injection of normal mouse serum (○) has no effect on platelet counts.

vity. There was a progressive increase in anti-platelet activity from Day 9 (OD at 405 nm: 1071 ± 182; uninfected controls: 326 ± 58) until the death of the animals.

Acute thrombocytopenia can be triggered by passive transfer of serum from *P. berghei*-infected, thrombocytopenic CBA mice

In order to study *in vivo* functional effect of serum anti-platelet factors in *P. berghei*-infected mice, the following transfer experiment was performed. Sera were obtained from 20 normal and 28 significantly thrombocytopenic (platelet counts: $0{\cdot}264 \pm 0{\cdot}099 \times 10^6/\mu l$) *P. berghei*-infected CBA mice. Circulating immune complexes were elevated in the thrombocytopenic mice (150 ± 29 ng aggregated mouse IgG/ml), whereas the controls had less than 20 ng/ml, as measured by the C1q binding assay. Normal mouse serum was treated identically and used as control. 0·5 ml of 1:2 diluted serum was injected i.v. in normal syngeneic recipients (five mice per group). Within minutes following the injection of malaria-infected, thrombocytopenic mouse serum, platelet counts fell from $1020 \pm 0{\cdot}028$ to $0{\cdot}645 \pm 0{\cdot}99 \times 10^6/\mu l$ in recipient mice (Fig. 5). Their platelet counts returned to normal values two days after serum transfer. In contrast, injection of normal mouse serum did not induce any change in platelet counts of recipient mice.

Production of monoclonal anti-platelet antibodies from *P. berghei*-infected mice

In order to demonstrate the production of anti-platelet antibodies during malarial infection, spleen cells were obtained from *P. berghei*-infected mice and fused with NS-2 myeloma cells. Proliferating hybrids were screened using the platelet monolayer ELISA described above. Six IgG monoclonal antibodies were produced and assessed as anti-platelet by using the platelet monolayer ELISA. None of these monoclonal antibodies were able to recognize *P. berghei* antigens in both ELISA and immunofluorescence assays. The potential effect of these monoclonal antibodies on platelets *in vivo* was evaluated. 0·5 ml of culture supernatants was injected i.v. in normal CBA mice and the platelet counts followed up. Four monoclonal antibodies (CV 6.9, CV 5.4, CV 5H.7, and CV 5H.8) were able to induce a significant but transient thrombocytopenia within minutes after injection and one of them, CV 5H.7, platelet counts remained low for 1 hr (Fig. 6). In contrast anti-platelet mAbs CV 6.2 and CV 6.4 had no effect on platelets *in vivo*.

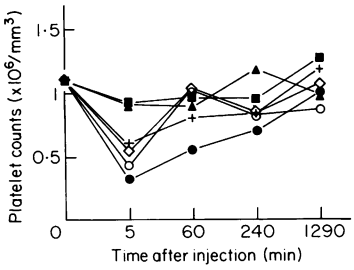

Figure 6. Effect of i.v. injection of monoclonal anti-platelet antibodies (mAb) derived from spleen cells of *P. berghei*-infected CBA/Ca mice. MAbs CV 5H.8 (+), CV 5.4 (○), CV 6.9 (◊) and CV 5H.7 (●) were able to induce a significant thrombocytopenia within minutes after injection, whereas mAbs CV 6.2 (■) and CV 6.4 (▲) had no effect.

DISCUSSION

In the present study, the mechanisms of malaria-induced thrombocytopenia were analysed in an experimental model.

First, as in human malaria, thrombocytopenia was associated with peripheral hyperdestruction of platelets since bone marrow megakaryocyte numbers were increased and platelet survival was reduced. These observations confirm those reported in malaria patients (Beale, Cormack & Oldrey, 1972; Horstmann *et al.*, 1981; Wilson *et al.*, 1982). Both reduced platelet production and increased platelet clearance could be involved in malaria-induced thrombocytopenia, as has been shown recently in autoimmune thrombocytopenic purpura (Ballem *et al.*, 1987). Some degree of dysthrombopoiesis cannot be ruled out by our studies. Increased peripheral platelet destruction may be due to several mechanisms, which can be classified in immune or non-immune. Non-immune mechanisms, such as direct interactions between infectious agents and platelets, have been described in several models (Wilson *et al.*, 1982), including penetration of the malaria parasite in blood platelets (Fajardo & Tallent, 1974). Disseminated intravascular coagulation (DIC) leads to thrombocytopenia in the context of immune and non-immune diseases (Salmon, Lambert & Hiernaux, 1968). Immune mechanisms potentially able to lead to platelet destruction comprise circulating immune complexes, *in situ*-formed immune complexes (i.e. at the level of the platelet membrane), and anti-platelet autoantibodies resulting from an interruption of immunological tolerance. Elevated levels of PAIgG are considered as an argument in favour of immune mechanisms (Kelton, 1983; Shaw *et al.*, 1984).

Second, data presented here suggest that helper T cells are involved in the pathogenesis of *P. berhei*-induced thrombocytopenia. This is supported by the prevention of thrombocytopenia in CD4$^+$ T-cell-depleted animals after treament with anti-CD4 monoclonal antibodies and by the occurrence of thrombocytopenia in *P. berhei*-infected ATxBM mice reconstituted with CD4$^+$ T-cell populations. The development of some degree of thrombocytopenia in *P. berghei*-infected ATxBM mice reconstituted with CD8$^+$ CD4$^-$ T cells (ATxBM.2$^+$ mice) can be explained in two ways. On the one hand, although the T-cell population used for the reconstitution was over 98% CD8$^+$ CD4$^-$ pure as judged by flow cytometry, it is possible that the few CD4$^+$ CD8$^-$ T cells transfered have had an effect *in vivo*. On the other hand, although treatment with anti-CD8 mAb had no protective effect on the thrombocytopenia, some effects of the CD8$^+$ CD4$^-$ T-cell population on the platelets cannot be ruled out. This possibility is under current investigation. The T-cell dependency indicates that, in this model, the thrombocytopenia is related to the immune response of the host. This T-cell dependency also argues against non-immune mechanisms, such as interactions between platelets and the infectious agent or parasite-induced disseminated intravascular coagulation (DIC) (Borochowitz, Crosley & Metz, 1970; Butler *et al.*, 1973). Furthermore, there was no detectable evidence of DIC in the thrombocytopenia associated with *P. berghei* infection in WAG rats, as plasma fibrinogen were normal, fibrinogen split products undetectable and heparin treatment unable to interfere with thrombocytopenia (G. E. Grau, unpublished observations). However, some degree of non-specific platelet clearance due to malaria-associated splenomegaly cannot be ruled out.

Third, the mechanisms by which CD4$^+$ T cell can affect platelets should be envisaged. Helper T cells may be involved via the humoral immune response, via the formation of anti-platelet antibodies and/or immune complexes or via the production of several lymphokines. The humoral immune response appears to be involved in the triggering of thrombocytopenia as serum anti-platelet antibodies were detectable during malaria infection and since spleen cells from malaria-infected animals were able to produce monoclonal anti-platelet antibodies. The anti-platelet antibody response developing during malaria infection was of low magnitude compared with general polyclonal activation. This could be due at least partly to the fact that the assay detects only free antibodies and that those bound to platelets might have been cleared. The role of circulating immune complexes cannot be ruled out in the thrombocytopenia of this model. The level of serum immune complexes was shown to be highly T-cell dependent, as it was significantly reduced in *P. berghei*-infected mice treated with anti-CD4 monoclonal antibodies (Grau *et al.*, 1986b). Immune complexes might be partly responsible for the induction of thrombocytopenia by passive transfer of serum from malaria-infected mice.

Finally, it is conceivable that besides modulating the production of antibodies, helper T cells might be involved in thrombocytopenia by the production of lymphokines. The role of the cytokine tumour necrosis factor/cachectin (TNF) in neurovascular complications of *P. berghei* infection has been documented (Grau *et al.*, 1987). Indeed, continuous infusion of recombinant mouse TNF in mice induced thrombocytopenia after 4 days (G. E. Grau and P. F. Piguet, unpublished observations). However, treatment of *P. berghei*-infected CBA mice with anti-TNF antibody prevented the occurrence of cerebral malaria but had no protective effect on thrombocytopenia. Thus, in this regard, although both thrombocytopenia and neurological syndrome are T-cell dependent complications of malaria infection, their pathogenesis appear to differ in terms of cytokine involvement. Other lymphokines produced by activated helper T cells may affect haemopoiesis and/or platelet functions and play a role in the triggering of malaria-associated thrombocytopenia. This possibility represents an open field for further studies.

In conclusion, in this experimental model, thrombocytopenia appears to be the expression of T-cell dependent immunopathological reactions of the malaria-infected host.

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