Effect of Amphotericin B on Parasitemia and Survival of *Plasmodium Berghei*-infected Mice

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Key Words

Red blood cells • Plasmodium • Phosphatidylserine • Eryptosis • Parasitemia

Abstract

Premature death of *Plasmodium*-infected erythrocytes is considered to favourably influence the clinical course of malaria. Amphotericin B has previously been shown to trigger suicidal erythrocyte death or eryptosis, which is characterized by cell membrane scrambling leading to phosphatidylserine exposure at the cell surface. Phosphatidylserine-exposing cells are rapidly cleared from circulating blood. The present study thus tested whether amphotericin B exerts a direct effect on *Plasmodium falciparum* and influences eryptosis of infected erythrocytes, parasitemia and host survival in murine malaria. To this end, human erythrocytes were infected *in vitro* with *Plasmodium falciparum* and mice were infected with *Plasmodium berghei* ANKA by *in vivo* intraperitoneal injection of parasitized murine erythrocytes (1×10^6). Half of the infected mice received amphotericin B (1.5 mg/kg b.w. i.v.) from the 8th day of infection. Amphotericin B

($\geq 1 \mu\text{M}$) compromised the intracellular development of the parasite in human erythrocytes as evident from *in vitro* growth and DNA amplification assays. Amphotericin B further augmented the eryptosis of infected human erythrocytes. The administration of amphotericin B to infected mice tended to delay the increase of parasitemia and significantly delayed host death. All nontreated mice died from malaria within 27 days. In contrast, some 50% of amphotericin B-treated mice survived for more than 27 days after infection. In conclusion, amphotericin B augmented the suicidal death of infected erythrocytes and delayed the lethal course of malaria in *Plasmodium berghei* infected mice.

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Introduction

The intraerythrocyte parasite *Plasmodium* triggers suicidal erythrocyte death or eryptosis [1, 2], which is characterized by cell membrane scrambling leading to phosphatidylserine exposure at the cell surface [3]. The

cell membrane scrambling is stimulated by increase in cytosolic Ca^{2+} activity [4] and/or ceramide [5]. Ca^{2+} enters following activation of Ca^{2+} -permeable cation channels [4, 6-10]. Besides its effect on cell membrane scrambling, an increased cytosolic Ca^{2+} concentration stimulates Ca^{2+} -sensitive K^{+} channels [11, 12] leading to cellular loss of KCl and osmotically obliged water [13].

Phosphatidylserine-exposing cells are recognized by macrophages [14, 15], phagocytosed [16, 17] and thus rapidly cleared from circulating blood [18].

Accelerated clearance of infected erythrocytes [19] may counteract the development of parasitemia [20]. Enhanced susceptibility to eryptosis and subsequently accelerated clearance of *Plasmodium*-infected erythrocytes presumably contribute to the relative protection against a severe course of malaria in carriers of sickle-cell trait, beta-thalassemia-trait, homozygous Hb-C and G6PD-deficiency [21-26], in iron deficiency [1] as well as following treatment with lead [2], chlorpromazine [27] or cyclosporine [28]. Erythropoietin may inhibit the cation channel [29] but by the same token may render erythrocytes more susceptible to eryptosis [30] and may again favourably influence the course of malaria [31].

Eryptosis is further stimulated by amphotericin B [32], which at least in theory, could be effective in malaria. As a matter of fact, administration of amphotericin B has indeed been suggested to positively influence the course of malaria [33-36], an effect, however, attributed to lysis of host erythrocytes in the trophozoite stage of *Plasmodium falciparum* [36] rather than to accelerated eryptosis.

The present study explored, whether amphotericin B exerts a direct effect on the parasite, augments the lysis or the suicidal death of *Plasmodium falciparum*-infected human erythrocytes and whether these effects correlate with a favourable influence on parasitemia and host survival during infection with *Plasmodium berghei*.

Methods and Materials

Human erythrocytes were drawn from healthy volunteers. The study was approved by the Ethical Commission of the University of Tübingen.

Animal experiments were performed in healthy SV129/J wild type mice (aged 4 months, both male and female). The animals had free access to standard chow (C1310, Altromin, Lage, Germany) and drinking water. Blood was drawn by incision of the tail vein. The animal experiments were performed

according to the German animal protection law and approved by the local authorities.

For infection of human erythrocytes the human pathogen *Plasmodium falciparum* (*P. falciparum*) strain BinH [37] was grown *in vitro* [38]. Parasites were cultured as described earlier [39, 40] at a hematocrit of 2% and a parasitemia of 2-10% in RPMI 1640 medium supplemented with Albumax II (0.5%; Gibco, Karlsruhe, Germany) in an atmosphere of 90% N_2 , 5% CO_2 , 5% O_2 [41, 42].

To estimate the *in vitro* growth of *Plasmodium falciparum* the BinH strain was cultured and synchronized to the ring stage by sorbitol treatment as described previously [43]. For the *in vitro* growth assay, synchronized parasitized erythrocytes were aliquoted in 96-well plates (200 μl aliquots, 1% hematocrit, 0.5-2% parasitemia) and grown for 48 h in the presence or absence of amphotericin B. The parasitemia was assessed at time 0 and after 48 h of culture by flow cytometry. Parasitemia was defined by the percentage of erythrocytes stained with the DNA/RNA specific fluorescence dye Syto16.

To estimate DNA/RNA amplification of the intraerythrocytic parasite, the culture was ring stage-synchronized [41] and re-synchronized after 6 h of culture (to narrow the developmental parasite stage), aliquoted (200 μl aliquots, 2% hematocrit and 10% parasitemia) and cultured for further 16 h in the presence or absence of amphotericin B. Thereafter, the DNA/RNA amount of the parasitized erythrocytes was determined by Syto16 fluorescence as a measure of intraerythrocytic parasite copies.

For infection of mice *Plasmodium berghei* ANKA-parasitized murine erythrocytes (1×10^6) were injected intraperitoneally [42, 44, 45]. Where indicated amphotericin B (1.5 mg/kg b.w. i.v.) was administered from the eighth day of infection. Blood was collected from the mice for 13 days after infection by incision of the tail. Parasitemia was determined by Syto16 staining in FACS analysis. *In vitro* experiments were performed at 37°C in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO_4 , 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4), 5 glucose, 1 CaCl_2 [46]. Amphotericin B was added to the NaCl Ringer at final concentrations varying from 0.001 to 10 μM (Sigma, Schnellendorf, Germany). For *in vitro* treatment, the final hematocrit was adjusted to 0.3%.

Hemolysis was determined after 24 h by measuring the absorbance of the culture medium at 405 nm. For determination of phosphatidylserine exposure, FACS analysis was performed as described [4]. After incubation in the presence or absence of amphotericin B, suspensions of *Plasmodium falciparum*-infected erythrocytes were stained with annexin V-APC (BD Biosciences Pharmingen, Heidelberg, Germany) and/or with the DNA/RNA specific dye Syto16 (Molecular Probes, Göttingen, Germany) to identify phosphatidylserine-exposing and infected erythrocytes, respectively. For annexin V-binding, erythrocytes were washed, resuspended in annexin V-binding buffer (Ringer solution containing 5 mM CaCl_2 , pH 7.4), stained with annexin V-APC (dilution 1:20), incubated for 20 min at room temperature and diluted 1:5 with annexin V-binding buffer. Syto16 (final concentration of 20 nM) was

Fig. 1. Effects of amphotericin B on intraerythrocytic amplification and *in vitro* parasitemia. A. *In vitro* parasitemia with *P. falciparum* in human erythrocytes as a function of the amphotericin B concentration (arithmetic means $\pm$ SEM, $n = 12$; three cultures were studied in quadruplicates). B. Intraerythrocytic DNA amplification as a function of the amphotericin B concentration (arithmetic means $\pm$ SEM, $n = 12$; three cultures were studied in quadruplicates). *** indicates significant difference ($p=0.001$) from absence of amphotericin B.

Fig. 2. Effects of amphotericin B on phosphatidylserine exposure of infected and noninfected human erythrocytes. Arithmetic means $\pm$ SEM ($n = 6$) of the percentage of annexin V-binding infected and noninfected erythrocytes following infection of human erythrocytes with *P. falciparum* at the indicated concentration of amphotericin B. **, *** indicate significant difference ($p \leq 0.01$, $p \leq 0.001$) from noninfected erythrocytes, ### indicates significant difference ($p \leq 0.001$) from absence of amphotericin B.

Fig. 3. Effects of amphotericin B on hemolysis. Arithmetic means $\pm$ SEM ($n = 3$) of the percentage of hemolysed infected (white bars) or non-infected (black bars) erythrocytes following infection with *P. falciparum* in the presence of amphotericin B at the indicated concentrations.

added directly to the diluted erythrocyte suspension or co-incubated in the annexin V-containing buffer solution. Erythrocytes were analyzed by flow cytometry (FACS-Calibur, Becton Dickinson, Heidelberg, Germany) in fluorescence channel FL-1 for Syto16 (detected at 530 nm) and in FL-4 for annexin V-APC fluorescence intensity (detected at 660 nm).

Data are expressed as arithmetic means $\pm$ SEM and statistical analysis was made by t-test or ANOVA using Tukey's test as post hoc test, as appropriate. $p < 0.05$ was considered as statistically significant.

Results

To explore whether amphotericin B exerts a direct effect on the parasite, the influence of amphotericin B on the *in vitro* growth of *Plasmodium* in human erythrocytes was determined. The infected erythrocytes were synchronized to the ring stage by sorbitol treatment. Within 48 hours the percentage of infected erythrocytes increased from 7.7% to 22.1% in the absence of amphotericin B.

Fig. 4. Effect of amphotericin B treatment on parasitemia of *Plasmodium berghei*-infected mice. A: Original histograms of parasitemia-dependent syto 16 fluorescence in untreated animals (upper panels) and animals treated from day 8 until day 20 with 1.5 mg/kg b.w. amphotericin B i.v. (lower panels) 10 (left panels) and 20 (right panels) days after infection with *P. berghei*. B: Arithmetic means $\pm$ SEM of parasitemia in mice without treatment (open circles, $n = 6-10$) or with 1.5 mg/kg b.w. of amphotericin B i.v. (closed circles, $n = 6$) as a function of days after infection with *P. berghei*. C: Survival of mice as in B as a function of days after infection with *Plasmodium berghei*.

Depending on the concentration employed, amphotericin B blunted the increase in the percentage of parasitized erythrocytes, an effect reaching statistical significance at $= 1 \mu\text{M}$ amphotericin B concentration (Fig. 1A). In addition, the presence of amphotericin B decreased the intraerythrocytic DNA amplification of the parasite, an effect reaching statistical significance at $1 \mu\text{M}$ amphotericin B concentration (Fig. 1B).

To determine the effect of infection and of amphotericin B on eryptosis, the percentage of phosphatidylserine-exposing erythrocytes was determined by measurement of annexin V-binding in FACS analysis. Infection with *P. falciparum* was followed by a steep increase in the percentage of annexin V-binding infected and noninfected erythrocytes (Fig. 2). The increase in annexin V-binding was significantly higher in infected than in noninfected erythrocytes (Fig. 2). Amphotericin B increased the phosphatidylserine exposure of infected and noninfected erythrocytes (Fig. 2), an effect reaching statistical significance at $5 \mu\text{M}$ amphotericin B.

Additional experiments were performed to unravel a hemolytic action of amphotericin B. To this end, the hemoglobin concentration was determined in the supernatant of erythrocytes following infection in the presence and absence of amphotericin B. As shown in Fig. 3, infection increased hemolysis, which was, however, not significantly augmented in the presence of amphotericin B up to a concentration of $10 \mu\text{M}$. Thus, amphotericin B augmented cell membrane scrambling of infected erythrocytes largely without disrupting the integrity of the cell membrane.

To determine the *in vivo* effect of amphotericin B, mice were treated with amphotericin B (1.5 mg/kg b.w. i.v.) from the 8th day of infection, i.e. at a time point, when parasitemia was still low (Fig. 4B). The percentage of infected erythrocytes increased to a similar extent with or without amphotericin B treatment. At any given day

the parasitemia tended to be lower in the amphotericin B treated mice than in animals without amphotericin B treatment, a difference, however, not reaching statistical significance (Fig. 4A, B).

In contrast, the treatment with amphotericin B resulted in enhanced survival *Plasmodium berghei*-infected mice. As shown in Fig. 4C, all untreated animals died within 27 days after the infection but some 50% of amphotericin B-treated animals survived the infection for more than 27 days.

To test whether stimulation of eryptosis contributes to the beneficial effect of amphotericin B treatment on the survival of *Plasmodium berghei*-infected mice, the

Fig. 5. Effect of amphotericin B treatment on phosphatidylserine exposure of infected and noninfected erythrocytes in *Plasmodium berghei*-infected mice. Arithmetic means $\pm$ SEM (n = 6) of the percentage of phosphatidylserine-exposing infected (right bars) and non-infected (left bars) erythrocytes in animals without (white bars) and with (black bars) amphotericin B treatment. *** indicates significant difference (p=0.001) from the absence of amphotericin B. ### indicates significant difference (p=0.001) from non-infected erythrocytes.

percentage of phosphatidylserine-exposing erythrocytes was determined on the 15th day of infection. As shown in Fig. 5, the percentage of phosphatidylserine-exposing infected erythrocytes was higher than that of non-infected erythrocytes. More importantly, amphotericin B treatment indeed enhanced the percentage of phosphatidylserine-exposing infected erythrocytes. The effect of amphotericin B was small and statistically not significant in noninfected cells, but marked and statistically significant in infected cells. Thus, amphotericin B potentiates the eryptotic effect of infection.

Discussion

The present observations reveal a favourable influence of amphotericin B treatment on the survival of *Plasmodium berghei* infected mice. Similar to earlier

observations [44], the infection of untreated mice with *Plasmodium berghei* took an invariably lethal course within 27 days. In contrast, some 50% of the amphotericin B-treated mice survived the infection more than 27 days. Those animals did, however, eventually die later. Thus, the effect of amphotericin B was sufficient to delay the lethal course of murine malaria but did not prevent a delayed death of the infected mice. Similarly, amphotericin B treatment delayed but did not halt the increase of parasitemia.

In theory, the positive effect of amphotericin B treatment could have been due to amphotericin B-induced lysis of the trophozoite stage of *Plasmodium falciparum* [36]. Moreover, amphotericin B may have accelerated the suicidal death of infected erythrocytes, leading to premature phosphatidylserine exposure of infected erythrocytes with subsequent engulfment by macrophages [16, 17] and clearance from circulating blood [18]. According to the *in vitro* experiments, amphotericin B directly compromised the intraerythrocytic development of the parasite at higher concentration.

Eryptosis is enhanced in some clinical conditions, including iron deficiency [18], sickle-cell anemia [47, 48], beta-thalassemia [26], glucose-6-phosphate dehydrogenase (G6PD)-deficiency [26], phosphate depletion [49], Hemolytic Uremic Syndrome [50], sepsis [51], malaria [52, 53] and Wilson's disease [54]. Moreover, eryptosis is triggered or modified by a wide variety of substances [3, 32, 55-60]. Several of the eryptosis-inducing diseases and xenobiotics have already been shown to influence the course of malaria, i.e. genetic erythrocyte disorders, such as sickle-cell trait, beta-thalassemia-trait, homozygous Hb-C and G6PD-deficiency [21-26], iron deficiency [1], lead [2], chlorpromazine [27], paclitaxel [61], and cyclosporine [28]. However; unlike amphotericin B, those diseases and substances substantially decrease parasitemia. At the *in vivo* concentrations employed amphotericin B did not significantly affect parasitemia. Possibly, the concentration required to trigger eryptosis and/or lysis, were not reached during the *in vivo* treatment at the chosen dosage. Alternatively, as phosphatidylserine exposing erythrocytes adhere to the vascular wall [48, 62-65], the parasitemia within circulating blood may not reliably reflect the number of infected erythrocytes. Nevertheless, the possibility must be considered that the protective effect may have been due to interference with other mechanisms, e.g. inhibition of inflammation. Clearly, additional experiments are needed to identify the underlying mechanism accounting for the effect on mouse survival.

In conclusion, in mice, amphotericin B delays the lethal course of *Plasmodium berghei*-induced malaria. At the dosage chosen, the favourable influence of amphotericin B on the survival of infected mice may not be due to toxicity to the pathogen or due to eryptosis or lysis of infected erythrocytes, but may involve further mechanisms decisive for the host response to infection.

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