Influence of Chlorpromazine on Eryptosis, Parasitemia and Survival of *Plasmodium berghei* Infected Mice

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Key Words

Malaria • Apoptosis • Cell death

Abstract

Chlorpromazine has previously been shown to trigger suicidal erythrocyte death or eryptosis, which is characterized by exposure of phosphatidylserine at the erythrocyte surface and cell shrinkage. Premature suicidal death of infected erythrocytes is in turn considered to delay development of parasitemia and thus favourably influence the clinical course of malaria. The present experiments have been performed to explore whether chlorpromazine influences *in vitro* parasite growth and eryptosis of *Plasmodium falciparum* infected human erythrocytes and *in vivo* parasitemia and survival of *P. berghei* infected mice. Phosphatidylserine was estimated from annexin V binding and cell volume from forward scatter in FACS analysis. *In vitro* infection of human erythrocytes increased annexin binding and decreased forward scatter, effects augmented in the presence of chlorpromazine ($\geq 10 \mu\text{M}$). Chlorpromazine did not significantly alter intraerythrocytic DNA/RNA content but significantly ($\geq 1 \mu\text{M}$) decreased *in vitro* parasitemia.

In chlorpromazine treated mice erythrocytes were more rapidly cleared from circulating blood than in nontreated mice. Parasitemia in *P. berghei* infected mice was significantly decreased (from 50 % to 28 % of circulating erythrocytes 22 days after infection) and mouse survival significantly enhanced (from 0 % to 80 % 30 days after infection) by addition of 1 mM chlorpromazine to the drinking water from the first day of infection. In conclusion, chlorpromazine favourably influences the course of malaria, an effect at least partially due to stimulation of suicidal erythrocyte death.

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Introduction

Chlorpromazine triggers eryptosis [1], the suicidal erythrocyte death characterized by cell shrinkage and by exposure of phosphatidylserine at the cell surface [2]. The phosphatidylserine exposure results from scrambling of cell membrane phospholipids, which is triggered by increased cytosolic Ca^{2+} activity [2, 3]. Moreover, cell membrane scrambling is sensitized to Ca^{2+} by ceramide, which is formed following activation of a sphingomyelinase

[4]. Cytosolic Ca^{2+} activity may be increased by Ca^{2+} entry through Ca^{2+} permeable cation channels which are activated by excessive cell shrinkage, oxidative stress and energy depletion [3, 5, 6]. Besides its ability to stimulate cell membrane scrambling, Ca^{2+} activates Ca^{2+} sensitive K^{+} channels [7], which leads to exit of KCl and osmotically obliged water and thus to cell shrinkage [8]. The stimulation of suicidal erythrocyte death parallels the ability of Ca^{2+} to trigger apoptosis of nucleated cells [9].

As macrophages are equipped with receptors specific for phosphatidylserine [10, 11], erythrocytes exposing phosphatidylserine at their surface will be rapidly recognized, engulfed and degraded [12, 13] and are thus expected to be rapidly eliminated from circulating blood.

On the other hand, premature suicidal erythrocyte death has been suggested to confer some protection against a severe course of malaria [14]. Sick cell trait, beta-thalassemia-trait, homozygous Hb-C and G6PD-deficiency lead to premature senescence and/or eryptosis upon infection with *Plasmodium* thus leading to accelerated clearance of ring stage-infected erythrocytes [15, 16]. The early clearance of infected erythrocytes prevents tissue sequestration of the late stage-infected erythrocytes and leads to removal of erythrocytes prior to the development of trophozoites with the ability to intoxicate macrophages [17].

Chlorpromazine has indeed been shown to be effective in the treatment of malaria [18].

The present experiments have been performed to explore whether chlorpromazine triggers phosphatidylserine exposure of infected erythrocytes and thus influences the clinical course of malaria.

Materials and Methods

Animals, cells and solutions

Animal experiments were performed according to German animal protection law and approved by the local authorities (registration number PY 2/06). Healthy SV129/J wild type mice (aged 4 months, both male and female) were used for the experiments. Mouse erythrocytes were drawn from animals by retroorbital venopuncture or by incision of the tail vein. Human erythrocytes were drawn from healthy volunteers.

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 . Chlorpromazine was added to the NaCl Ringer at final concentrations varying from 0.1 μM to 100 μM (Sigma, Taufkirchen, Germany). For *in vitro* chlorpromazine treatment, the final haematocrit was adjusted to 0.3 %.

Determination of phosphatidylserine exposure

FACS analysis was performed as described [3]. After incubation in the presence or absence of chlorpromazine, suspensions of noninfected erythrocytes were stained with annexin V-FLUOS (Roche, Mannheim, Germany), 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 binding, erythrocytes were washed, resuspended in annexin-binding buffer (Ringer solution containing 5 mM CaCl_2 , pH 7.4), stained with annexin V-APC (dilution 1:20) or annexin V-FLUOS (dilution 1:100), incubated for 20 min at room temperature, and diluted 1:5 with annexin binding buffer. Syto16 (final concentration of 20 nM) was added directly to the diluted erythrocyte suspension or co-incubated in the annexin binding buffer. Cells were analyzed by flow cytometry (FACS-Calibur, Becton Dickinson, Heidelberg, Germany) in FL-1 for Syto16 or annexin V-FLUOS fluorescence intensity (detected at 530 nm) and in FL-4 for annexin V-APC fluorescence intensity (detected at 660 nm).

In vitro cultivation of P. falciparum

For infection of human erythrocytes the human pathogen *P. falciparum* strain BinH [19] was grown *in vitro* [20] in human erythrocytes. Parasites were cultured as described earlier [21, 22] at a haematocrit 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 .

In vivo proliferation of P. berghei

For infection of mice *P. berghei* ANKA-parasitized mouse erythrocytes (1×10^6) were injected intraperitoneally [23] into wildtype mice with free access to drug free or chlorpromazine (1 mM) containing drinking water from the first day of infection. Blood was collected from the mice 8 days after infection by the incision of the tail. Parasitemia was determined by Syto-16 staining in FACS analysis.

In vitro growth assays of P. falciparum infected human erythrocytes

The *P. falciparum* BinH strain was cultured and synchronized to the ring stage by sorbitol treatment as described previously [24]. For the *in vitro* growth assay, synchronized parasitized erythrocytes were aliquoted in 96-well plates (200 μl aliquots, 1% haematocrit, 0.5–2 % parasitemia) and grown for 48 h in the presence or absence of chlorpromazine (0.1 μM –100 μM). 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, the culture was ring stage-synchronized, and re-synchronized after 6 h of culture (to narrow the developmental parasite stage), aliquoted (200 μl aliquots, 2% haematocrit and 10% parasitemia) and cultured for further 16 h in the presence or absence of chlorpromazine

Fig. 1. Effects of chlorpromazine on phosphatidylserine exposure of infected and noninfected erythrocytes and on intraerythrocytic parasite growth. A. Dot blots of annexin binding and Syto16 fluorescence of human erythrocytes in noninfected cells (upper panels) and in cells infected with *P. falciparum* (lower panels) following a 48 hours incubation in the absence (left panels), or presence (right panels) of 10 µM chlorpromazine. B. Arithmetic means $\pm$ SEM (n=6) of annexin binding of infected (closed symbols) and noninfected (open symbols) erythrocytes before and after treatment with chlorpromazine as a function of the chlorpromazine concentration. * indicates significant difference (p<0.05) from absence of chlorpromazine, # indicates significant difference to noninfected erythrocytes. C. Intraerythrocytic DNA amplification in erythrocytes as a function of the chlorpromazine concentration (arithmetic means $\pm$ SEM, n=6). D. *In vitro* infection of human erythrocytes with *P. falciparum* as a function of chlorpromazine concentration (arithmetic means $\pm$ SEM, n=6). * indicates significant difference (p<0.05) from absence of chlorpromazine.

(0.1 µM –100 µM). Thereafter, the DNA/RNA amount of the parasitized erythrocytes was determined by Syto 16 fluorescence as a measure of intraerythrocytic parasite copies.

Measurement of *in vivo* clearance of fluorescence-labelled erythrocytes

Fluorescence-labelled erythrocytes from mice were obtained by staining erythrocytes with carboxyfluorescein diacetate, succinimidyl ester (CFSE) from Molecular Probes (Leiden, The Netherlands). The labelling solution was prepared by addition of adequate amounts of a CFSE stock solution

(10 mM in DMSO) to phosphate-buffered saline (PBS) to yield a final concentration of 5 µM. Then, the cells were incubated with labelling solution for 30 min at 37°C. The cells were pelleted at 400 g for 5 min, washed and resuspended in fresh, prewarmed PBS. The fluorescence-labelled erythrocytes were injected into the tail veins of healthy SV 129 mice. At indicated time points, blood was taken from the mice and CFSE- dependent fluorescence intensity of the erythrocytes was measured in the fluorescence channel FL-1. In mice injected with infected CFSE-labelled erythrocytes, the mice were sacrificed 2 hours after injection and the spleens were dissected and carefully mashed through a net. Finally the CFSE-dependent fluorescence inten-

Fig. 2. Effects of chlorpromazine on forward scatter of infected and noninfected erythrocytes. A. Dot blots of forward scatter and syto16 fluorescence of noninfected human erythrocytes (upper panels) and of *P. falciparum*-infected human erythrocytes (lower panels) following a 48 hours incubation in the absence (left panels), or presence (right panels) of 10 μ M chlorpromazine. B. Arithmetic means $\pm$ SEM (n=6) of forward scatter of infected (closed symbols) and noninfected (open symbols) human erythrocytes as a function of the chlorpromazine concentration. * indicates significant difference (p<0.05) from absence of chlorpromazine, # indicates significant difference to noninfected erythrocytes.

sity of the erythrocytes in the spleen suspensions was measured in FL-1. The percentage of CFSE-positive erythrocytes was calculated in % of the whole cell population.

Statistics

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

Results

Phosphatidylserine at the cell surface was estimated from annexin V binding in FACS analysis. The percentage of annexin-binding erythrocytes in freshly drawn blood

Fig. 3. Clearance of erythrocytes from untreated or chlorpromazine treated mice from circulating blood. Disappearance of CFSE labelled non infected and infected mouse erythrocytes from circulating blood following injection into the tail vein of untreated and chlorpromazine (100 μ M in drinking water) treated mice. A: Arithmetic means $\pm$ SEM (n = 6) of the percentage of CFSE-labelled noninfected erythrocytes in circulating blood of untreated (open symbols) or chlorpromazine (closed symbols) treated animals. * indicates significant difference (p<0.05) from untreated animals. B: Arithmetic means $\pm$ SEM (n=6) of the percentage CFSE labelled infected erythrocytes in circulating blood of untreated (open symbols) or chlorpromazine treated (closed symbols) animals. * indicates significant difference from the untreated animals. C: Abundance of CFSE labelled untreated (open bar) or chlorpromazine treated (closed bar) erythrocytes in spleen. Arithmetic means $\pm$ SEM (n=6). * indicates significant difference from the untreated animals.

Fig. 4. Parasitemia and survival of *P. berghei* infected mice. A: Original histograms of parasitemia in untreated animals (upper panels) and animals treated from day 10 until day 22 with 1 mM chlorpromazine (lower panels) 10 (left panels) and 22 (right panels) days after infection with *P. berghei*. B: Arithmetic means $\pm$ SEM of parasitemia in mice without treatment (open circles, n=12) or with 1 mM chlorpromazine (closed circles, n=10) as a function of days after infection with *P. berghei*. * indicates significant difference from the untreated animals. C: Survival of mice without treatment (open circles) or with 1 mM chlorpromazine (closed circles) as a function of days after infection with *P. berghei*.

was low (1.48 ± 0.05 %, n=6). A 24 hour incubation in Ringer solution increased the percentage of annexin binding cells to 5.28 ± 0.29 % (n=6), a 48 hour incubation in Ringer solution increased the percentage of annexin binding noninfected erythrocytes to 14.93 ± 0.39 % (n=6). As shown in Fig. 1, infection significantly enhanced the percentage of annexin binding erythrocytes. The effect of infection was significantly enhanced in the presence of chlorpromazine (Fig. 1B). The effect reached statistical significance at 10 μ M chlorpromazine. Thus, chlorpromazine potentiated the scrambling effect of infection on phosphatidylserine exposure.

Intraerythrocytic DNA amplification in erythrocytes was not significantly influenced by chlorpromazine at the concentrations employed. *In vitro* growth of *Plasmodium falciparum* in human erythrocytes was, however, significantly decreased at chlorpromazine concentrations of $\geq 1 \mu$ M.

Infection further decreased erythrocyte forward scatter. Chlorpromazine led to a moderate but significant further decrease of forward scatter, pointing to chlorpromazine induced cell shrinkage (Fig. 2).

A next series of experiments explored, whether the enhanced phosphatidylserine exposure accelerates clearance of erythrocytes from circulating blood *in vivo*. For this purpose mice were either left untreated or were treated with 100 μ M chlorpromazine in drinking water, their erythrocytes were labelled with the dye CFSE and re-injected into the tail vein. As illustrated in Fig. 3A, the clearance of erythrocytes in chlorpromazine treated mice was slightly, but significantly faster than erythrocyte clearance in untreated mice. Most importantly, the decline was particularly fast in infected CFSE labelled erythrocytes (Fig. 3B). On the other hand, the fraction of labelled erythrocytes recovered in the spleen was significantly enhanced by chlorpromazine treatment after 120 minutes. (Fig. 3C).

Further experiments were performed to elucidate the impact of chlorpromazine treatment on the course of malaria infection *in vivo*. To this end mice were infected with *P. berghei*. As shown in Fig. 4, parasitemia was less than 10 % 8 days after infection but gradually increased the next 14 days. In untreated animals, the percentage of parasitized erythrocytes increased up to 50 %. The addition of 100 μ M chlorpromazine had only a slight effect on parasitemia (data not shown).

Thus, the concentration of chlorpromazine was increased to 1 mM chlorpromazine, which indeed significantly blunted the increase of parasitemia.

The effects of chlorpromazine on the parasitemia was paralleled by enhanced survival of the chlorpromazine treated animals. Whereas all nontreated animals died within 30 days after the infection, 80% of the chlorpromazine treated animals survived the infection for more than 30 days. Thus, chlorpromazine treatment indeed significantly modified the course of malaria.

Discussion

The present experiments confirm the ability of chlorpromazine to induce eryptosis [1]. The concentrations required for the effect are above the plasma concentrations observed during treatment of psychiatric patients, which usually remain between 0.1 and 1 μM [25] but have been reported to increase up to 5 μM [26].

The ability of chlorpromazine to trigger scrambling of the cell membrane is shared by several amphiphiles [27].

Chlorpromazine further decreased forward scatter pointing to cell shrinkage, which was presumably due to increase of cytosolic Ca^{2+} activity, activation of Ca^{2+} sensitive K^+ (GARDOS) channels [8], K^+ exit, hyperpolarization of the erythrocyte membrane potential and Cl^- exit [28]. The exit of KCl is followed by osmotically obliged water and thus leads to cell shrinkage [29]. Chlorpromazine has further been reported to induce echinocytic shape [30] and cell swelling [31].

As shown earlier [32, 33], eryptosis is further stimulated by *P. falciparum* infection. Intraerythrocytic *Plasmodia* impose oxidative stress to the host cell, which in turn activates the Ca^{2+} permeable cation channels [24]. The following Ca^{2+} entry leads to stimulation of cell membrane scrambling. Moreover, oxidation may favour cell membrane phosphatidylserine scrambling [34].

Macrophages are equipped with receptors specific for phosphatidylserine [10, 11] and thus engulf and degrade erythrocytes exposing phosphatidylserine at their surface. Accordingly, chlorpromazine treated phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood. The majority of cleared labelled cells were accumulated in the spleen (Fig. 3C). Notably, to the extent that eryptosis precedes hemolysis, the erythrocyte is engulfed by macrophages prior to disruption of the cell membrane and thus eryptosis

prevents the release of intracellular proteins including haemoglobin into the plasma.

Besides its effect on eryptosis, chlorpromazine exerted a remarkable inhibitory effect on *in vitro* parasitemia. Again, the concentrations required for this effect may be achieved *in vivo*. This effect does not correlate with the scrambling effect of chlorpromazine and is not paralleled by decreased intraerythrocytic survival of the pathogen but may be an effect on parasite invasion. As both, host cell and parasites, were exposed to chlorpromazine, the impaired parasite entry could have resulted from an effect of chlorpromazine on the parasite or the host cell or both. Nevertheless, the sustained intraerythrocyte survival of the parasites demonstrates that chlorpromazine, at the employed concentrations, does not appreciably affect the intraerythrocytic survival of the pathogen.

Most importantly, chlorpromazine proved to be similarly effective *in vivo*. Following infection with *P. berghei* the parasitemia increased significantly less rapidly in chlorpromazine treated mice than in untreated mice. For this effect, 1 mM chlorpromazine had been added to the drinking water, which at a fluid intake of 4 ml/day corresponds to a daily intake of 1.2 mg. The dosage per kg body weight would be higher in mice ($\approx 40 \text{ mg/kg bw}$) than in humans (3–10 mg/kg bw), the dosage per metabolic and fluid turnover would, however, be similar. At a daily water intake of one liter in humans, 1 mM in drinking water would lead to an uptake of 1 mmol, corresponding to a daily dose of some 300 mg, which is well in the range of the dosages (150–600 mg) used for psychiatric patients [25]. The concentrations achieved under those conditions may trigger eryptosis in less than 10% of the infected cells. Thus, chlorpromazine delays, but does not completely prevent the gradual increase of parasitemia.

Decreased *in vivo* parasitemia following chlorpromazine treatment may be expected to affect the survival of infected animals. Similar to previous series [23], none of the untreated mice survived the infection with *P. berghei*. In contrast, 80 % of the chlorpromazine treated mice were still alive 30 days after the infection. Thus, chlorpromazine treatment indeed favourably modified the course of malaria.

The protective effect of chlorpromazine probably results from the decreased ability of pathogens to enter noninfected erythrocytes leading to delayed development of parasitemia. Moreover, the scrambling effect of chlorpromazine decreases the life span of infected erythro-

cytes in circulating blood. A similar eryptotic effect has been observed in iron deficiency [35] and following application of several chemicals such as cordycepin [36], Bay-5884 [37], curcumin [38], methylglyoxal [39], amyloid peptides [40], listeriolysin [41], paclitaxel [42], valinomycin [43], cyclosporine [44], lead [45], mercury [46] and copper [47]. As a matter of fact iron deficiency [32] and treatment with lead [33] similarly accelerate eryptosis of infected erythrocytes and are thus similarly protective against a severe course of malaria.

In conclusion, chlorpromazine impairs the entry of parasites into erythrocytes and accelerates eryptosis

of infected erythrocytes, effects, which may favourably influence the course of malaria.

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