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Artemether and Artesunate Show the Highest Efficacies in Rescuing Mice with Late-Stage Cerebral Malaria and Rapidly Decrease Leukocyte Accumulation in the Brain▽

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The murine model of cerebral malaria (ECM) caused by *Plasmodium berghei* ANKA (PbA) infection in susceptible mice has been extensively used for studies of pathogenesis and identification of potential targets for human CM therapeutics. However, the model has been seldom explored to evaluate adjunctive therapies for this malaria complication. A first step toward this goal is to define a treatment protocol with an effective antimalarial drug able to rescue mice presenting late-stage ECM. We evaluated the efficacy of artemisinin, artemether, artesunate, and quinine given intraperitoneally once a day, and combinations with mefloquine, in suppressing PbA infection in mice with moderate parasitemia. Artemether, artesunate, and quinine were then evaluated for efficacy in rescuing PbA-infected mice with ECM, strictly defined by using objective criteria based on the presentation of clinical signs of neurological involvement, degree of hypothermia, and performance in a set of six motor behavior tests. Artemether at 25 mg/kg presented the fastest parasite killing ability in 24 h and fully avoided recrudescence in a 5-day treatment protocol. Artemether and artesunate were equally effective in rescuing mice with late-stage ECM (46 and 43% survival, respectively), whereas quinine had a poor performance (12.5% survival). Artemether caused a marked decrease in brain leukocyte accumulation 24 h after the first dose. In conclusion, artemether and artesunate are effective in rescuing mice with late-stage ECM and decrease brain inflammation. In addition, the described protocols for more strict clinical evaluation and for rescue treatment provide a framework for studies of CM adjunctive therapies using this mouse model.

The *Plasmodium berghei* ANKA (PbA) murine model of cerebral malaria (ECM) is the most widely used animal model for human CM. This model has been intensely explored to study mechanisms of CM pathogenesis, and the role of several mediators on disease development has been unraveled using different interventions such as mediator blockade by monoclonal antibodies and gene knockout (a list of interventions is provided in the supplemental file on reference [7]). However, and surprisingly, the model has seldom been explored to assess the efficacy of potential adjunctive therapies aimed to improve survival in combination with antimalarial drugs. Indeed, although nearly 300 papers have been published using this model in the past 3 decades, only a handful have used approaches that can be characterized as adjunctive therapy interventions, with the administration of an ancillary treatment in addition to a primary antimalarial drug to mice presenting clinical signs of ECM (5–7). This shortcoming has helped to fuel criticisms on the relevance of ECM as a model for the human disease (38; debated in references 7, 15, 29, 31, and 33).

Assessments of adjunctive therapies for CM in this model will require basic data on the efficacy of different antimalarial drugs themselves in rescuing mice with established clinical signs of ECM, and such data are scarce. Prada et al. treated PbA-infected mice with chloroquine or artemether and reported >95% efficacy for both drugs, but the criterion for treatment was the level of parasitemia rather than the presentation of neurological signs (27). Reis et al. also showed high efficacy (100%) of chloroquine and artesunate in preventing the syndrome, also at early stages of disease when mice presented slight decreases in SHIRPA scores but apparently no clinical signs of ECM (28). Baptista et al. reported that a single dose of pyrimethamine given 15 to 20 h before the expected onset of ECM (on day 5 of infection with mice presenting a mean parasitemia of ca. 4%) was able to prevent the syndrome (3). Dai et al., on the contrary, showed that chloroquine treatment starting at an early stage of ECM (parasitemia > 7%, low activity levels, and body weight loss > 2%) resulted in 70% mortality and did not prevent cognitive and motor deficits in the survivors (8). Golenser et al. tried several treatment protocols with quinine and dihydroartemisinin (DHA), and basically early treatments prevented the neurological syndrome delaying mortality, whereas later treatments were much less effective (11). Subcurative doses of quinine or DHA given on days 4 to 6 also prevented ECM (22). Artesunate and artemisone have also been shown to be effective in preventing the development of ECM in mice when given before, or at early stages of, ECM development (12, 36).

To test the efficacy of an ECM-rescuing therapy, it is necessary to establish well-defined clinical parameters of neurological involvement in order to clearly determine the stage of the disease at which the treatment is administered. Cognitive, sensorimotor function and behavior evaluations, as provided for instance by the SHIRPA and other well-established proto-

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cols (17, 20), are very useful in helping to establish a comprehensive clinical status of mice. The presentation of well-recognized clinical signs of ECM such as ataxia, limb paralysis, convulsions, moderate to severe hypothermia, loss of righting reflex, and/or coma should in principle be the guide for treatment decisions in experimental protocols, however it must be emphasized that these signs usually appear only at very late stages of disease, in mice with full-blown ECM. Bienvenu et al. defined five stages of clinical presentation in mice receiving treatment: 0, no symptoms; 1, ruffled fur (non-ECM specific); 2, motor impairment; 3, respiratory distress; and 4, coma. In that study, when the first dose of artesunate or the combination artesunate-erythropoietin was given on day 6, ca. 70% of the mice were at stages 1 or 2, and most survived. We have previously defined a protocol for late-stage (rescue) treatment of mice with ECM using artemether (6). To treat mice, we used as the basic criterion the expression of well-established clinical signs of full-blown murine ECM: ataxia, limb paralysis, convulsions, loss of righting reflex, and/or coma. Because even in the presence of these classical ECM-associated neurological signs the overall clinical status of the mice may vary considerably (e.g., mice with ataxia are obviously in better shape than mice in coma), we added a composite clinical score based on a set of six simple, fast-to-perform, motor behavior tests (transfer arousal, locomotor activity, tail elevation, wire maneuver, contact righting reflex, and righting in arena) derived from the SHIRPA protocol (17, 20), as well as rectal temperature, to better define the overall clinical status of each mouse at the time of treatment. We have shown that artemether at 50 mg/kg given intraperitoneally (i.p.) was able to rescue 32% of mice presenting late-stage ECM. However, because we were evaluating the adjunctive effect of the calcium channel blocker nimodipine, mice receiving artemether also received the nimodipine vehicle containing ethanol and polyethylene glycol (PEG), which therefore probably influenced the results.

In the present study we defined the efficacy of a number of antimalarial drugs (quinine, artemisinin, artesunate, artemether, and combinations artemether-mefloquine) in (i) clearing PbA parasitemia with treatments starting on day 5 of infection and (ii) rescuing mice with late-stage ECM. In addition, we evaluated the effect of artemether in decreasing leukocyte sequestration in the brain 24 h after the first dose in mice with ECM. We expect this study to be useful in helping to define standards for experiments designed to evaluate adjunctive therapies for CM using the PbA model.

MATERIALS AND METHODS

Mice, parasites, and infection. Eight- to ten-week-old C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were housed in groups of no more than five per cage with free access to chow and water. Mice were allowed to adapt to their new environment for 3 days before experimentation. All experimental protocols were reviewed and approved by the La Jolla Bioengineering Institute (LJBI) Institutional Animal Care and Use Committee. The *Plasmodium berghei* ANKA PbA-GFPcon 259cl2, which is a genetically modified parasite of clone cl15cy1 of the ANKA strain that expresses green fluorescent protein (GFP) constitutively during the whole life cycle, was used (a kind donation of MR4, Manassas, VA; deposited by C. J. Janse and A. P. Waters; MR4 reagent number MRA-865). The parasite was propagated in C57BL/6J mice, and in each experiment a fresh blood sample was obtained from a passage mouse and a suspension containing 10^6 parasitized red blood cells (pRBC) in 100 μl was injected i.p. in each mouse of the experimental groups. Parasitemia, motor behavior, and rectal temperature were checked beginning on day 5 after infection. Parasitemia was checked by using flow cytometry and quantified by counting the number of pRBC in 10,000 RBC. After treatment, thin blood smears were made from a drop of tail blood and stained with Giemsa to distinguish dead from viable parasites. We considered as dead parasites those that we could no longer clearly distinguish the typical morphology and presented themselves as a mass of condensed matter inside the RBC, many times featured just as a dark dot. The slides were examined under a light microscope at ×1,000 magnification with an oil immersion lens (Nikon Eclipse E200). Parasitemia was calculated by counting the number of pRBC in at least 1,000 RBC.

Clinical assessment. A set of six simple behavioral tests (transfer arousal, locomotor activity, tail elevation, wire maneuver, contact righting reflex, and righting in arena) adapted from the SHIRPA protocol (17, 20) was used to provide a better estimate of the overall clinical status of the mice during infection. The performance in each test was assessed by using a modified scoring system (0 to 5 for transfer arousal, 0 to 4 for locomotor activity, 0 to 4 for tail elevation, 0 to 4 for wire maneuver, 0 to 3 for contact righting reflex, and 0 to 3 for righting in arena), and a composite score was built ranging from 0 to 23, where 23 indicates maximum performance and 0 indicates complete impairment (usually coma). Body temperature was monitored by using an Accorn Series Thermocouple thermometer with a mouse rectal probe (Oakton Instruments, Vernon Hills, IL). ECM was defined as the presentation of one or more of the following clinical signs of neurological involvement: ataxia, limb paralysis, poor righting reflex, seizures, rollover, and coma.

Experimental design. The present study was divided in three parts. (i) The first part was the treatment of PbA-infected mice on day 5 of infection. At this time point, infected mice show a moderate, rising parasitemia but present no ECM signs, which usually start to manifest on day 6. Treating mice at this point allowed us to evaluate the efficacy and rapidness of parasite killing, parasite clearance, and recrudescence avoidance by different antimalarial drugs and doses. (ii) The second part of the study was the treatment of PbA-infected mice presenting clinical signs of ECM. In this way, we sought to determine the efficacy of suitable treatment protocols defined in part i in rescuing mice with late-stage ECM. (iii) The third part of the study was the quantification of leukocyte accumulation in brain vessels before and after treatment of mice with ECM in order to determine the effect of treatment on brain inflammation.

Antimalarial drug regimens. (i) Treatment before ECM development. We had previously shown that artemether at 50 mg/kg, coadministered with a solution of ethanol and PEG 400 in saline (1:1:8 [vol/vol], the vehicle for nimodipine), was able to rescue one-third of the mice with late-stage ECM (6). For the present study, we defined half this dose (25 mg/kg) as our reference for artemether itself. Artemisinin was given at this same dose, and artesunate was given at a molar-equivalent dose (32 mg/kg) and also at twice this amount (64 mg/kg). On day 5 postinoculation, infected mice were treated with one of the following drug regimens. (i) Artemether (Artesiane [20 mg/ml], kindly provided by Dafra Pharma, Belgium) was already prepared in coconut oil and was administered i.p. at 25 mg/kg. (ii) Artesunate (Sigma, St. Louis, MO) was dissolved in 5% sodium bicarbonate in saline and administered i.p. at 32 or 64 mg/kg. (iii) Artemisinin (Sigma) was dissolved in 10% dimethyl sulfoxide (DMSO) and then 90% saline, mixed thoroughly, and administered i.p. at 25 mg/kg. (iv) Quinine (Sigma) was dissolved in saline and delivered i.p. at 60 or 120 mg/kg. These dosages are within the range of dosages commonly used in rodent studies with *P. berghei* (5, 32, 40). Mice treated with artemisinin, artemether, artesunate, or quinine received treatment once a day for 5 days.

Artemether was also administered in combination with mefloquine for either a 1- or a 3-day treatment. For the 1-day treatment, artemether at 25 mg/kg was administered i.p., together with mefloquine at 40 mg/kg i.p., or per gavage, single dose on day 5 of infection. For the 3-day treatment, artemether was given for 3 days (once a day) i.p. starting on day 5 of infection, and mefloquine was administered together with the third dose of artemether by oral gavage. In both cases, mefloquine (Sigma) was dissolved in 10% DMSO and 90% saline.

(ii) Treatment after ECM development (rescue treatment). Treatments with artemether (25 mg/kg), artesunate (32 mg/kg), or quinine (120 mg/kg) were administered only after infected mice were diagnosed with ECM, using the following strict criteria: (i) presentation of at least one well-recognized clinical sign of neurological involvement in ECM, i.e., ataxia, limb paralysis, convulsions, rollover, loss of righting reflex, and coma; (ii) moderate to severe hypothermia (rectal temperature $< 33°C$); and (iii) poor motor behavior performance (composite score < 10). Each injection subsequent to the initial dose was given in the morning.

Quantification of leukocyte accumulation in the brain. Ten mice presenting clinical signs of ECM and with similar parasitemias, body temperatures, and composite motor scores were randomly assigned to two groups. In the first group, five mice were euthanized, and the brains were collected and processed for

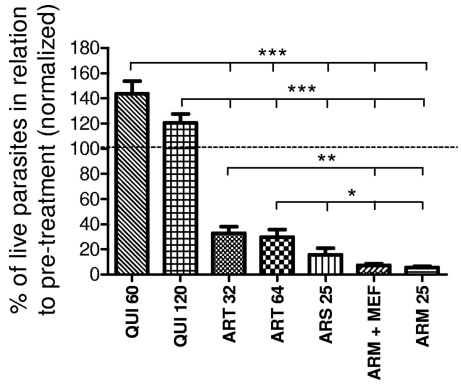

FIG. 1. Relative decay of viable circulating parasites 24 h after one dose of artemether (ARM), artesunate (ART), artemisinin (ARS), quinine (QUI), or artemether-mefloquine (ARM + MEF), in relation to normalized parasitemia on day 5 (just before treatment = 100%, indicated by the dashed line [actual pretreatment parasitemia values for each mouse of each group can be seen in Fig. 3]). The numbers after each drug code on the *x* axis refer to the drug concentrations in mg/kg. Artemether, with or without mefloquine, showed the highest efficacy, with initial parasitemia reduced by more than 95%. Quinine, at either 60 or 120 mg/kg, was unable to curb parasite growth 24 h after a single dose. The results are means ± the SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

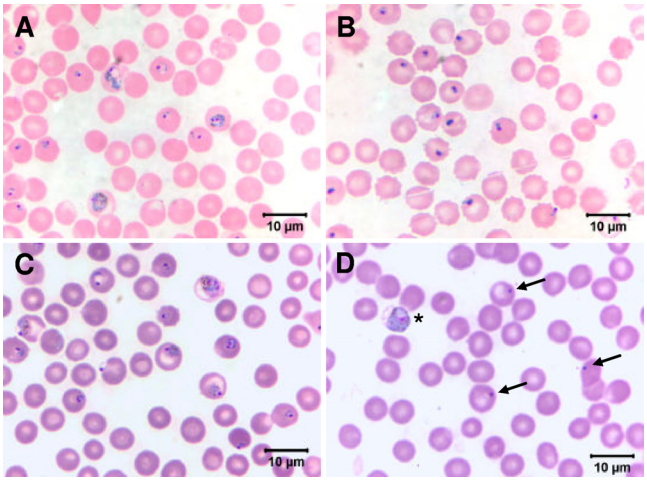

FIG. 2. Giemsa-stained blood thin smears of PbA-infected mice just before (A and C) and 24 h after (B and D) the first dose of artemether at 25 mg/kg. Note that in panel B most parasites appeared as condensed dark masses or dots, as opposed to the healthy morphology observed just before treatment (A), with parasites at different maturation stages showing clearly defined blue cytoplasm and round, red nuclei. In panel D, three condensed dots are seen (arrows), as well as a viable form suggestive of a gametocyte (asterisk), compared to the healthy morphology observed just before treatment (C).

histology. In the second group, five mice were treated with artemether at 25 mg/kg; 24 h later the four mice alive were also euthanized, and the brains were collected and processed for histology. Brains from two uninfected mice were also collected and served as controls. Mice were anesthetized (ketamine, 150 mg/kg; xylazine, 10 mg/kg) and perfused with 5 ml of heparinized saline (10 U/ml), followed by 5 ml of 4% paraformaldehyde (PFA), both solutions at a rate of 1 ml/min, using a gravity perfusion setup. Each brain was carefully collected immediately after perfusion and stored in PFA during 48 h for fixation. Brains were cut in four coronal slices of 2 to 3 mm using a mouse brain blocker (David Kopf Instruments, Tujunga, CA), and slices were numbered 1 to 4 (with slice 1 being the frontal lobe/olfactory bulb and slice 4 being the cerebellum-brainstem). Each slice was embedded in paraffin, and 5-μm sections were obtained at approximately 400-μm intervals (four sections per slice). Sections were mounted in glass slides and stained with hematoxylin-eosin (H&E). Leukocyte quantification was performed on section 2 of each slice; therefore, a total of four sections were counted per mouse. The number of leukocytes in each vessel of each section was quantified by using an ocular grid calibrated with a ×400 magnification (field dimensions, 200 by 180 μm) in a Nikon microscope (Eclipse E200). The whole area of each section was similarly quantified with the grid calibrated at ×40 magnification. Quantification was performed by an experienced investigator in a blinded fashion. Pictures were taken with a SPOT RT3 camera (Diagnostic Instruments, Inc.; 1,014 by 721 pixels).

Statistical analyses. Results were expressed as means and standard errors of the mean (SEM) unless otherwise stated. Statistical analysis were performed using one-way or two-way analysis of variance with Tukey's or Bonferroni's post tests when comparing whether one or two parameters, respectively, varied among the different treatment groups. A log-rank test was used to compare the different survival curves. A *P* value of <0.05 was considered significant.

RESULTS

Efficacy of antimalarial drugs in clearing PbA parasitemia on day 5 of infection. Before evaluating the efficacy of different antimalarial drugs in rescuing mice with ECM, we performed a preliminary trial to evaluate their efficacy and rapidity in clearing PbA parasitemia. We chose to treat mice on day 5, which is a time point when mice present moderate, rising parasitemia but usually no neurological involvement, and therefore we expected that all drugs, even the slower-acting ones, would be effective in clearing parasitemia without mortality, allowing the definition of doses, speediness of parasite clearance, and length of treatment to avoid recrudescence. We chose quinine, artemisinin, and its derivatives artesunate and artemether since these are the mainstream drugs used to treat human CM. On attempting to define a simpler and faster treatment protocol, we also evaluated combinations of a fast-acting/rapidly cleared drug (artemether) in combination with a slower-acting/slowly cleared drug (mefloquine).

Figure 1 shows the efficacy of each drug regimen in killing parasites within 24 h after the first dose. As expected, artemisinin and its derivatives showed the highest efficacy. Artemether at 25 mg/kg (with or without mefloquine) was significantly more effective than any other regimen, killing ca. 95% of parasites in 24 h. Figure 2 depicts the morphology of parasites just before and 24 h after a single dose of artemether at 25 mg/kg. Total parasite clearance with artemether was obtained already at 48 h, and no recrudescence was observed after a 5-day treatment regimen (Fig. 3A). We also tried a shorter (3-day) protocol with artemether at 25 mg/kg, but then recrudescence was observed (data not shown). Artesunate given i.p. at doses equivalent to (32 mg/kg) or twice (64 mg/kg) the artemether dose was also able to clear parasites in 48 h. However, recrudescence was observed with both dosages, especially at 32 mg/kg (Fig. 3B and C). Artemisinin at 25 mg/kg in DMSO-PEG showed a pattern of efficacy and recrudescence similar to artesunate at 32 mg/kg (Fig. 3D). Quinine at 60 or 120 mg/kg i.p. was unable to cause immediate decrease in parasitemia, which actually kept increasing in the first 24 h. Afterward, a 50% reduction in parasitemia was observed but without further decreased with the 60-mg/kg dose. The 120-mg/kg dose was effective and eventually cleared the parasites, but recrudescence was observed after the last dose (Fig. 3E and F). In addition, quinine at this higher dose was not well tolerated.

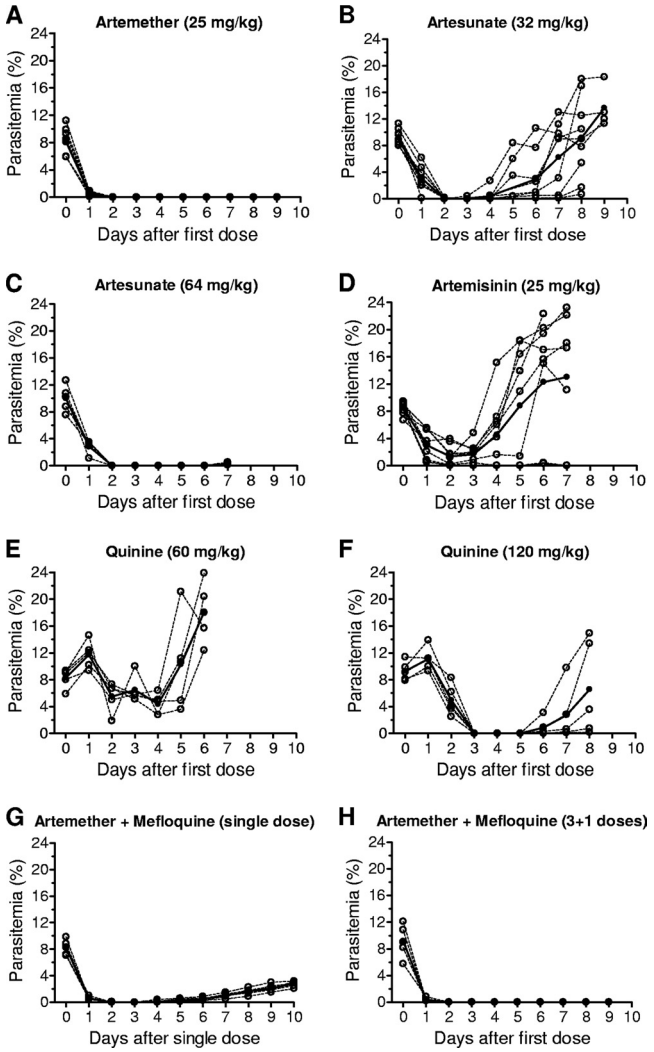

FIG. 3. Individual (dashed lines) or mean (solid lines) curves of parasitemia in PbA-infected mice after treatment with different antimalarial drugs and regimens. All treatments started on day 5 of infection. (A to F) Single-drug treatments, administered i.p., once a day for 5 days; (G) artemether i.p. plus mefloquine per gavage, single dose; (H) artemether i.p. once a day for 3 days + mefloquine per gavage, single dose together with a third artemether dose.

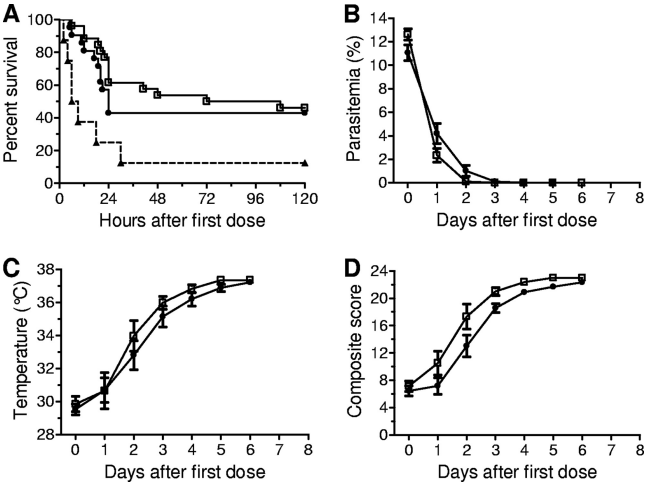

FIG. 4. Efficacy of artemether at 25 mg/kg ($n = 25$), artesunate at 32 mg/kg ($n = 21$), and quinine at 120 mg/kg ($n = 8$) in rescuing mice with late-stage ECM. The results for artemether and artesunate were pooled from three independent experiments. Only one experiment was performed for quinine. (A) Survival curves: artemether-treated (□) and artesunate-treated (●) mice showed similar survival rates, which were significantly superior to quinine-treated mice (▲) ($P < 0.05$). The survival rates between experiments varied from 36 to 56% for artemether and from 38 to 50% for artesunate. (B, C, and D) Profiles of parasite clearance (B), body temperature (C), and motor behavior scores (D) in ECM-survivor mice after a 5-day course of treatment with artemether or artesunate (quinine was not included since only one of eight animals survived). The results in panels B, C, and D are means ± the SEM.

After receiving injections, the mice presented a transitory decrease in activity and signs of discomfort.

Antimalarial drug combination therapy. As described above, the only drug able to prevent recrudescence after a 5-day treatment was artemether at 25 mg/kg. Other effective drugs, such as artesunate, require additional doses to prevent recrudescence in case an experiment is designed with follow-ups longer than 5 days. We attempted to simplify the treatment scheme with a single-dose treatment using artemether (intended to provide fast initial clearance of the parasites in the first critical hours when mice are most likely to die of ECM) in combination with mefloquine, an antimalarial drug with slower action but prolonged bioavailability. When artemether at 25 mg/kg and mefloquine at 40 mg/kg were given simultaneously i.p., parasitemia decreased sharply in the first 24 h, similar to artemether alone, and remained low thereafter. However, treated mice visibly worsened right after injection, showing signs of discomfort, and despite the decrease in parasitemia, all mice died 48 to 96 h after the single-dose injection (data not shown). We then tried mefloquine at 40 mg/kg per gavage in association with artemether i.p., both with a single dose at time zero. This protocol was well tolerated by the mice and showed good efficacy, with complete clearance of parasites between 48 and 72 h. However, recrudescence occurred in all mice at 120 h (Fig. 3G). Finally, artemether at 25 mg/kg i.p. for 3 days, with a single dose of mefloquine at 40 mg/kg per gavage, together with the third artemether dose, resulted in complete clearance and no recrudescence up to 240 h, when the mice were euthanized (Fig. 3H).

Efficacy of antimalarial drugs in rescuing mice with established clinical signs of ECM. In view of the efficacy results obtained in the first phase of the study, we moved to evaluate the ECM-rescuing efficacy of artemether at 25 mg/kg i.p., artesunate at 32 mg/kg i.p., and quinine at 120 mg/kg i.p. Mice were followed up parasitologically and clinically by checking the performance in six motor behavior tests, body temperature, and manifestation of the classical clinical signs of ECM. When mice manifested one or more of the clinical signs of ECM, they were given one of the antimalarial drugs. The groups receiving either artemether, artesunate, or quinine did not differ in terms of parasitemia, composite clinical score, or body temperature at the time of the first dose (Fig. 4B to D), except for the quinine group that showed a significantly higher body temperature compared to artesunate-treated mice but not compared to artemether-treated mice. All treated mice were at a

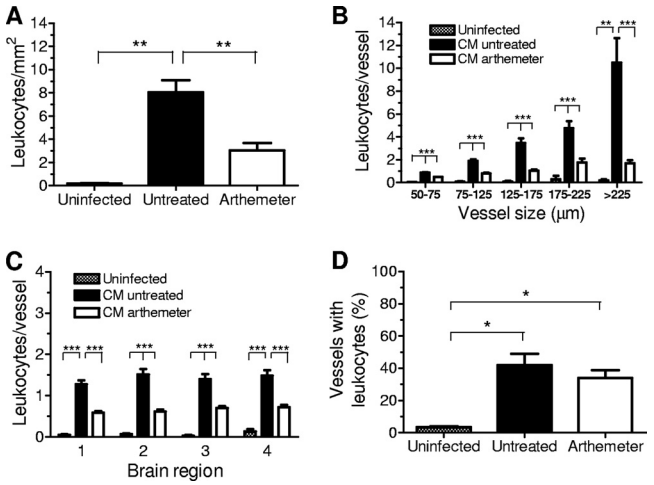

FIG. 5. Inhibition of inflammation in the brain of PbA-infected mice with ECM 24 h after a single dose of artemether at 25 mg/kg. (A) The number of intravascular leukocytes per mm^2 of brain area was markedly decreased 24 h after artemether treatment. (B) The decrease in the number of leukocytes was observed in vessels of all sizes (because during tissue sectioning the vessels are intercepted at a variety of angles, the vessel size was defined according to the maximum length, not diameter). (C) Vessels from the four regions of the brain analyzed (region 1 being anterior [frontal lobe] and region 4 being posterior [cerebellum-brainstem]) showed decreases in the number of leukocytes upon treatment. (D) The proportion of vessels containing leukocytes was not significantly different before or 24 h after treatment. The results are the mean ± the SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

late stage of ECM. Artemether and artesunate showed similar survival rates (46% versus 43% [not significant]), and most deaths occurred in the first 24 h in both groups (Fig. 4A). Conversely, quinine showed a poor performance, with only a 12.5% survival rate. This difference in survival was probably related to the difference in the capacity of each drug to kill the parasites, since artemether and artesunate acted much faster than quinine (Fig. 1). Again, artemether was more effective than artesunate in killing parasites in the first 24 h (Fig. 4B). There were no significant differences in the pace of recovery from hypothermia and motor impairment in survivor mice treated with either artemether or artesunate (Fig. 4C and D).

Artemether markedly decreases leukocyte accumulation in brain vessels. ECM is associated with marked leukocyte adherence and accumulation in brain vessels (4). We sought to determine whether rapid killing of the parasites would also lead to a fast downregulation of brain inflammation by quantifying the number of adherent leukocytes just before and 24 h after treatment with artemether. Indeed, the numbers of leukocytes per vessel and per mm^2 were markedly decreased 24 h after treatment (Fig. 5A). The decrease in leukocyte adherence was observed in vessels of all sizes (Fig. 5B) and in all regions of the brain (Fig. 5C). Interestingly, there was no difference in the percentage of vessels with leukocytes between treated and untreated mice (Fig. 5D). Representative images of leukocyte accumulation in brain vessels in the different groups are shown in Fig. 6.

DISCUSSION

In the present study we showed that artemether and artesunate were the most effective drugs in rescuing PbA-infected mice with late-stage ECM. Their efficacy was closely related to their ability to rapidly and effectively kill the parasites in the first 24 h of treatment. In addition, we have shown that artemether treatment markedly decreases brain inflammation in mice with ECM.

There have been only a few studies on rescue treatments of

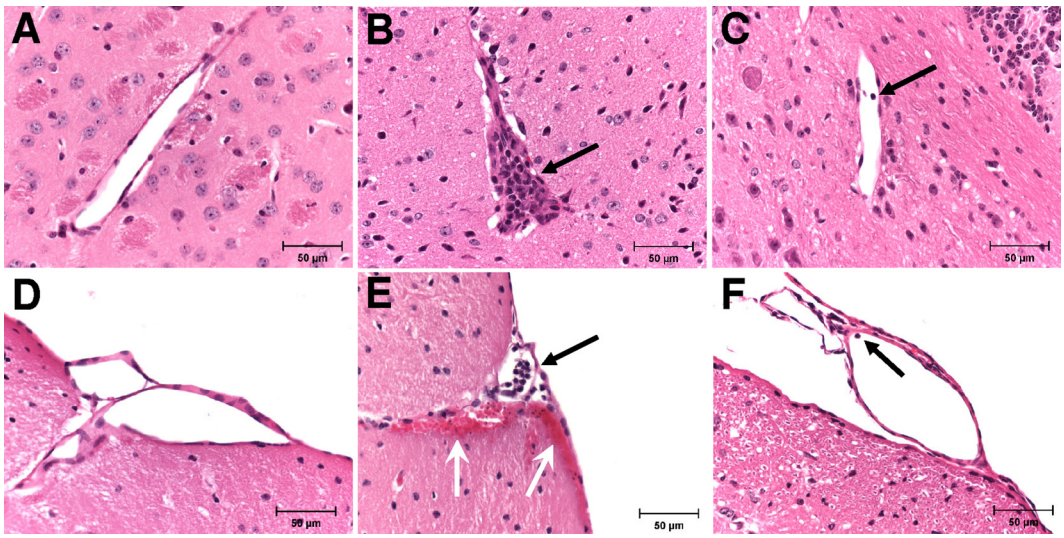

FIG. 6. Inhibition of inflammation in the brain of PbA-infected mice with ECM 24 h after a single dose of artemether at 25 mg/kg. (A and D) Parenchymal (A) and pial (D) vessels of uninfected control mice, showing no accumulation or adhesion of leukocytes; (B and E) parenchymal (B) and pial (E) vessels of untreated mice with ECM, plugged with leukocytes (black arrows); an extensive subarachnoid hemorrhage is seen in panel E (white arrows); (C and F) parenchymal (C) and pial (F) vessels of artemether-treated mice showing a remnant adherent leukocytes (black arrows). Magnitude of leukocyte accumulation and adherence was heterogeneous among vessels in both untreated and artemether-treated mice. All sections were stained with H&E (magnification, ×400).

ECM using antimalarial drugs in murine models (5, 6, 8, 11, 12, 27, 28, 36), and in most such studies treatment was administered before neurological signs were patent or at an early stage of ECM development. Quinine and artemisinin derivatives are the drugs of choice to treat human CM (16, 21) and therefore were chosen to be evaluated for ECM-rescuing efficacy in the PbA model. As expected, artemisinin and its derivatives artemether and artesunate showed the fastest parasite clearance effect. Artemether presented the best performance at a lower dose, which is likely related to the formulation, as oil-based formulations of artemisinin derivatives have been shown to provide a slower and more sustained drug release due to the "depot" effect than water-based formulations, which are cleared faster (19, 23).

Artemether was also the only drug able to fully prevent recrudescence following a 5-day treatment protocol. A 3-day treatment protocol is also possible with artemether as long as an oral dose of mefloquine is given together with the third dose of artemether, and this can be a convenient alternative for a shorter treatment. Our attempts for a single-dose treatment protocol failed, since the combination of i.p. artemether-mefloquine per gavage resulted in late recrudescence, and artemether i.p.-mefloquine i.p. turned out to be toxic, resulting in initial discomfort for the animals and later death despite parasite clearance. It is possible that the i.p. injection of mefloquine at 40 mg/kg resulted in overdose and the observed toxicity, which did not happen when the same dose was given per gavage. Mefloquine can present a number of toxic effects when given at high doses, potentially affecting the central nervous system and the gastrointestinal tract (2, 34). A similar effect, though less severe, may have occurred with quinine given i.p., since mice also showed discomfort upon injection of the higher dose (120 mg/kg). It is possible that an effective single dose treatment can still be attained with different drug combinations/dosages and delivery systems. Arteether, for instance, was shown to present better bioavailability, lower toxicity, and higher efficacy against *P. berghei* in rats when formulated with cremophore compared to sesame oil formulation (18). The use of a hybrid molecule derived from artesunate and mefloquine, named MEFAS, presented higher efficacy against *P. berghei* than the individual drugs given separately or combined (9). Woodard et al. showed that the monomeric trioxane fluoroanilide 4b (an artemisinin derivative) at a single dose as low as 6.8 mg/kg, combined with mefloquine at 20 mg/kg, was able to clear PbA parasitemia and prevent recrudescence in 80% of treated mice, whereas the combination artemether-mefloquine at the same single doses did not prevent recrudescence and resulted in 100% lethality (39). However, it should be noted that treatment was given only 24 h after infection, when parasitemia was presumably very low. Rottmann et al. recently reported that spiroindolones can be more effective than the artemisinins at lower doses (10 mg/kg) and were able to completely clear *P. berghei* parasitemia with a single higher dose (100 mg/kg) (30).

In the present study we did not evaluate the toxicity of the different drugs and dosages, which can be a concern since artemisinin derivatives, particularly those formulated in oil such as artemether, have been shown to cause neurotoxicity in animal models when given intramuscularly at relatively high doses for several days (1, 10, 18, 24). This concern is boosted by the fact that, in our case, mice being treated already present affected brains. Arteether or artemether given at 25 mg/kg (same dose as in the present study) daily for 7 days caused adverse effects in the brains of rats (1, 18). On the other hand, the same dose of artemether given daily for 28 days in mice produced no evident brain changes, although higher doses did (24). Because we used the i.p. route, a shorter (5-day) treatment scheme, and treatments were given to infected mice with ECM rather than to uninfected mice, a specific study would be necessary to determine whether these treatment schemes, under these circumstances, result in toxicity.

We have previously shown that artemether given i.p. at 50 mg/kg was able to rescue 32% of mice with late-stage ECM (6). However, in that study the concomitant injection of a vehicle containing ethanol and PEG may have influenced the results. We show here that artemether at half that dose (25 mg/kg) was able to rescue 46% of mice with late-stage ECM. Artesunate, at an equivalent molar dose (32 mg/kg), presented a similar efficacy (43%). On the other hand, quinine at 120 mg/kg showed very low efficacy (12.5%). These data suggest that both artemether and artesunate are appropriate drugs to be used in ECM-rescuing therapy experiments in the PbA model. The criteria we defined for starting the treatments were fairly strict, and only mice with late ECM presentations were included in the study. At this stage, untreated mice are likely to die within a few hours (17). Therefore, it is remarkable that artemether and artesunate were still able to rescue ca. 40% of the animals at this stage. This effect is likely due to the fast parasite-killing capacity of these drugs, which kill 70 to 95% of the parasites in 24 h, whereas quinine, unable to decrease parasitemia in the first 24 h, was ineffective.

In humans, artemether, artesunate, and quinine are mainstream drugs used to treat CM. Despite the fact that artemisinin derivatives clear parasitemia much faster, several studies failed to detect an obvious benefit of intramuscular artemether over intravenous quinine to rescue patients with CM (13, 16, 21). Nevertheless, intravenous artesunate has been shown to be superior to intramuscular artemether, with improved survival outcomes (25). The lower efficacy of artemether has been blamed on pharmacokinetics variability among patients due to slow and erratic absorption with relatively low conversion to the active metabolite dihydroartemisinin compared to artesunate, rather than on intrinsic differences in parasite-killing efficacy (14, 25). Indeed, in some patients, the oil-based artemether can remain on the site of injection not reaching proper plasma levels, resulting in a lack of efficacy. In rats, arteether in sesame oil achieves only a 20% bioavailability after a single intramuscular dose, and nearly 40% of the dose is retained in the muscle (18). We have not performed pharmacokinetic evaluations in the present study, but when using i.p. injections artemether was as effective in rescuing mice with ECM as artesunate; therefore, the shortcomings of intramuscular injections were not apparent with the i.p. injection in mice. On the other hand, this protocol was not appropriate for quinine. In humans, plasma levels of quinine are usually attained with an intravenous loading dose and maintained with short-interval injections (16). In our case, the 24-h period between the first and second doses was probably too long. Once-a-day treatments with similar doses of quinine have been shown to be effective when started right after parasite inoculation, when

parasitemias are low (32). However, a single daily dose when the parasitemia is moderate and rising was shown here to be insufficient to prevent disease progression. In contrast to artemisinin derivatives, quinine acts only on mature trophozoites and schizonts; hence, a discontinued dosage probably killed only a fraction of all parasites, leaving behind younger parasites to grow and replicate.

The ability of artemether in rescuing mice with ECM was associated with a marked decrease in leukocyte accumulation in brain vessels in the first 24 h, after a single dose. These data indicate that the inflammatory process associated with ECM is not sustained without parasite multiplication and is quickly downregulated as parasites are killed, with leukocyte migration to the brain (4) probably being inhibited. Similarly, limiting parasite growth with subcurative doses of quinine or DHA given on days 4 to 6 decreased the parasite burden and also decreased $CD8^+$ T-cell accumulation in the brain by 50% on day 6 (22). A different picture was apparent with pyrimethamine treatment, since a single dose given on day 5 of infection was reported to inhibit parasite growth without altering $CD8^+$ T-cell accumulation in the brain (3). However, it should be noted that, although no significant difference was detected, the number of $CD8^+$ T cells in the brain of pyrimethamine-treated mice was on average 40% lower than in vehicle-treated mice with ECM on day 7 (3), and therefore it is plausible that this trend might become evident in a larger sample size. In addition to their antiplasmodial effects, artemisinin derivatives present intrinsic anti-inflammatory properties (37), and this may also help to explain the reduced leukocyte accumulation in brain vessels in treated mice. Human CM is also associated with inflammation (35), and anti-inflammatory, immunomodulatory strategies have been proposed as potential alternatives for adjunctive treatment of CM (11). Our data also suggest that caution should be taken in human postmortem studies when interpreting findings of minor leukocyte accumulation in brain vessels, since most patients that die of CM usually receive antimalarial treatment for several hours before death (7, 26, 38).

In conclusion, artemether and artesunate are effective in rescuing mice with late-stage ECM and decrease brain inflammation. In addition, the present study describes a simple model for experiments designed to evaluate adjunctive therapies in ECM caused by PbA, with an accurate and yet simple characterization of the clinical status of sick mice by checking the expression of classical neurological signs, simple motor behavior assessment, and body temperature and with simple treatment protocols using artemisinin derivatives.

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