Selection and Characterization of Toxoplasma gondii Mutants Resistant to Artemisinin

Randolph L. Berens, Edward C. Krug,* Paul B. Nash,* and Tyler J. Curiel

Division of Infectious Diseases, University of Colorado Health Sciences Center, Denver, Colorado

Toxoplasma gondii infection, like malaria, is sensitive to inhibition by artemisinin (ART). Mechanisms of action for ART in malaria treatment have been proposed, but little is known about its effects in T. gondii infection. To better understand its inhibitory effects on T. gondii, mutants resistant to ART were selected by progressive culture in permissive levels of the drug. Five clonal isolates were established and characterized. The isolates were ~65-fold less sensitive to ART than is the parental RH and showed cross-resistance to the ART derivatives dihydroartemisinin and artemether. In addition to ART resistance, 3 clone (C9) formed morphologically unusual parasitophorous vacuoles and another (A2) was avirulent for mice and protected mice from challenge with the wild type. These clonal T. gondii mutant isolates will be useful for the study of not only the mechanism of action of ART but also parasite vacuole biology and virulence factors.

Infection with Toxoplasma gondii is a significant cause of morbidity and mortality in immunocompromised hosts, including those infected with the human immunodeficiency virus. Current anti-Toxoplasma drugs have significant toxicities, which may compromise anti-Toxoplasma or antiretroviral therapy. Thus, there is great need for alternative agents.

Studies from this [1] and other laboratories [2] have shown that T. gondii tachyzoites are sensitive to growth inhibition by artemisinin (qinghaosu, ART) or its derivatives. ART is a sesquiterpene lactone with an unusual endoperoxide bond [3] that is, along with several derivatives, very effective against chloroquine-resistant strains of Plasmodium falciparum [3–5]. Although a mechanism of action for ART in malaria treatment has been proposed [6], its mode of action in T. gondii infection is unknown. Mutants of T. gondii have been used previously to study the mechanism of action of several anti-Toxoplasma agents [7, 8]. Using similar methods, we derived mutant lines of T. gondii resistant to ART. Reported here are the results of the selection, isolation, and partial characterization of several ART-resistant clonal lines.

Materials and Methods

Materials. ART, dihydroartemisinin (DHART), deoxygenartemisinin (DESART), and artemether (MEART) were gifts of Hasuer, Inc. (Boulder, CO). All other reagents were from Sigma (St. Louis) except bovine serum (Gemini, Calabasas, CA), plasticware (Corning, Corning, NY), rabbit anti-Toxoplasma polyclonal antibody (Dako, Carpinteria, CA), and female ICR mice (Harlan Sprague-Dawley, Indianapolis).

Parasite growth. The RH isolate of T. gondii was maintained in and harvested from human fibroblasts as previously described [1, 9]. Human fibroblasts (HF) were grown in VA-13 [1] medium supplemented with 10% adult bovine serum for growth of noninfected fibroblasts or 0.3% bovine (Cohn Fraction V) serum albumin for infected fibroblasts. Harvested tachyzoites were enumerated using a hemacytometer. Parasites were frozen, stored, and thawed as described [10].

Drug stocks. ART and its derivatives were made as 20 mg/mL DMSO (dimethylsulfoxide) stocks, aliquotted, and stored at −20°C. Fresh aliquots were used for each study. Pyrimethamine was made as a 5-mg/mL DMSO stock. In all studies, the DMSO concentration was <1%.

Plaque assay. Confluent HF monolayers were infected with 200 fresh tachyzoites/well in 24-well plates and incubated in duplicate for 3 h before addition of test compounds or with pyrimethamine (10 µg/mL) as a positive drug control. Plates were incubated for 120 h and then methanol-fixed. Plaques were scored using a “4+” scale as described [1]. For mutant growth rate estimations, plates were infected as above and incubated until the plaque size was that of RH (4+) at 120 h. Differences in growth rates were estimated by comparing the time for the mutant and RH to reach 4+. Plaque-forming units and plating efficiency were determined as described [8].

ELISA quantitation. ELISA was done as described [11] in 96-well culture plates with confluent HF monolayers. Test compound was added 3 h after infection with 2000 tachyzoites/well. At 72 h after initial infection, plates were examined by phase-contrast microscopy to estimate approximate concentrations for 0%, 50%, and 100% growth inhibition and then methanol-fixed. The plates were washed and developed by sequential addition of rabbit anti-
Toxoplasma antibody diluted 1:20,000, horseradish peroxidase–conjugated goat anti–rabbit IgG, and o-phenylenediamine dihydrochloride as described [11]. The optical density was read at 450 nm. Results were averaged, and percent inhibition was calculated and plotted versus compound concentration. The concentrations resulting in 50% (IC₅₀) and 90% inhibition (IC₉₀) were determined graphically.

Selection of mutants. Two different procedures were used. For chemical mutagenesis, infected HF were exposed to either N-methyl-N’-nitro-N-nitrosoguanidine (MNNG) or ethylmethane sulfonate as described [7]. For selection of mutants by progressive culture in ART, cultures were started at an ART concentration of 0.2 µg/mL. The ART concentration was increased each time parasites displayed reproducible growth and infectivity after three sub-passages at that drug concentration. Clonal isolates were derived by limiting dilution in HF cells in 96-well plates [12].

Pathogenicity studies. Groups (3 each) of female ICR mice weighing 19–23 g were used. All mice were infected by intraperitoneal injection of 2000 freshly isolated tachyzoites [13]. Impression smears were made of the peritoneal fluid of dying mice and examined by phase-contrast microscopy for tachyzoites. Mice that survived 45 days after test inoculation were then challenged with RH tachyzoites and observed for 45 additional days.

Results

Attempts were made to establish resistant mutants using ethylmethane sulfonate or MNNG, but neither mutagen resulted in establishment of resistance using an ART selection range of 0.4–4 µg/mL. However, exposure to either mutagen resulted in pyrimethamine-resistant mutants capable of growth in 25 µg/mL pyrimethamine, 100 times the RH IC₉₀ (data not shown).

By progressive culture in ART, however, a culture growing in 35 µg/mL ART was established over a period of 9 months. At this concentration, growth of the mutants was ~5% that of RH, and ART was significantly toxic for HF. Since the mutant population growing in 25 µg/mL ART had an acceptable doubling time and because there were no signs of HF toxicity, 25 µg/mL was chosen as the concentration for further work.

Based on plaque assay, the uncloned mutant line growing in 25 µg/mL ART was ~50-fold less sensitive to ART than RH and grew approximately half as fast. No significant abnormalities were seen in mutant tachyzoite morphology by phase-contrast microscopy or hematoxylin-eosin staining.

Twenty-three clonal lines of the resistant culture were established by limiting dilution, 5 of which were chosen for further study. The plating efficiency of the clones was ~50%, equal to that of RH [8]. Four clones, designated A2, H9, A4, and G23, had normal morphology and growth rates relative to RH of 40%, 50%, 60%, and 60%, respectively.

One clone (C9) had a growth rate of 60% but formed enlarged, atypical parasitophorous vacuoles. Normally there is little space between the tachyzoites and the vacuole membrane, but in C9 there was a large separation between it.
and the parasites. Within 24 h of infection, the resulting parasitophorous vacuole increased to a size comparable to the HF nucleus, while containing as few as 2 tachyzoites.

The ART resistance of each clone was compared with RH (figure 1). All clones were ~65-fold more ART-resistant than was the parental RH. The average IC$_{50}$ and IC$_{90}$ values for the 5 clones were 6.6 µg/mL (23.4 µM) and 25.2 µg/mL (89.2 µM), respectively, compared with IC$_{50}$ and IC$_{90}$ values of 0.10 µg/mL (0.36 µM) and 0.38 µg/mL (1.3 µM) for RH. Each clone was also tested for resistance to the ART derivatives DHART and MEART, which have significant anti-Toxoplasma activity [1]. The respective IC$_{50}$ and IC$_{90}$ values for DHART for A2 (figure 2) were 3.8 µg/mL and 10.1 µg/mL versus 0.11 µg/mL and 0.23 µg/mL for RH. MEART values were 1.6 µg/mL and 4.0 µg/mL for the A2 compared with 0.04 µg/mL and 0.09 µg/mL for RH. The other clones showed similar resistance profiles, with IC$_{50}$ and IC$_{90}$ values essentially identical to those found for A2 (data not shown).

Both RH and the clones were tested for sensitivity to DESART [14]. RH, the clones, and HF were not inhibited by 50 µg/mL DESART. All clones showed parental sensitivity to pyrimethamine (data not shown).

The C9 clone showed the same atypical parasitophorous vacuole in the presence of DHART or MEART as for ART. Exposure to DESART resulted in normal parasitophorous vacuoles.

The effect of verapamil on ART-resistant clones was tested because it increases chloroquine efficacy in multidrug-resistant *P. falciparum* malaria [15]. However, incubation in the presence of 20 µM verapamil (a subinhibitory concentration [2]) did not affect the activity of ART (data not shown).

Each clone was tested for virulence in mice, and all except for A2 caused fatal toxoplasmosis in 8–11 days, with a clinical course identical to that of RH. Tachyzoites were present in peritoneal fluid of all dying mice. Mice infected with A2 showed no signs of toxoplasmosis for up to 45 days after infection, when they were challenged with 2000 fresh RH tachyzoites. These mice showed no signs of toxoplasmosis for up to 45 additional days after challenge with RH. These tests were repeated three times with identical results. However, 2000 A2 tachyzoites injected peritoneally into SCID mice killed them in ~8 days.

The 5 clones were cultured in the absence of ART for 30 days and then placed back in 25 µg/mL ART. After a growth lag of ~48 h, all clones resumed growth in the presence of drug identical to that of cultures continuously on drug and had virulence in mice as was described above. Of interest, the C9 clone formed normal parasitophorous vacuoles when cultured in the absence of ART but reformed enlarged vacuoles when

Figure 2. Comparative inhibition of artemisinin (ART) and artemisinin derivatives dihydroartemisinin (DHART) and artemether (MEART) in wild type (WT) and A2 clone.
placed back in drug. All the clones were stable to freezing and storage in liquid nitrogen for at least 6 months.

**Discussion**

ART and certain of its derivatives appear to be potential new anti-*Toxoplasma* agents. As a system for future research, ART-resistant mutants of *T. gondii* were obtained by exposure to progressively higher concentrations of drug. Chemical mutagenesis, which was successfully used to produce resistance to pyrimethamine, did not yield ART resistance. The uncloned ART-resistant line was ~50-fold less sensitive to ART than was the parental RH. The mutants cloned from this population were ~65-fold less sensitive to ART than was RH and showed cross-resistance to the ART derivatives DHART and MEART. Neither RH nor the clones were sensitive to DESART (ART with a reduced endoperoxide bond), suggesting that, as for *Plasmodium* species, the presence of the endoperoxide bond is required for the inhibitory effects of ART and derivatives on *Toxoplasma* species [6, 14].

None of the clones showed cross-resistance to the antifolate pyrimethamine, and incubation of the clones with ART in the presence of verapamil did not affect the activity of ART, suggesting that the resistance of these clones is specific for ART and its derivatives and does not involve increased drug efflux. These clones will be useful in understanding the mechanism of action of ART in *Toxoplasma* species.

Ten RH tachyzoites are lethal for mice [13], whereas 2000 A2 tachyzoites were not, demonstrating the avirulence of A2. Inoculation with A2 protected mice against RH challenge, suggesting that A2 elicits protective immunity. Study of the differences between A2 and RH may shed light on *T. gondii* virulence.

The C9 replicated in a normal parasitophorous vacuole when cultured in the absence of ART but formed the enlarged atypical vacuole when placed back on drug. The genetic changes resulting in ART resistance in the C9 appear to be linked with the physiology of the parasitophorous vacuole. This linkage may aid in vacuole studies.

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**References**