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Phenotypic Changes in Artemisinin-Resistant *Plasmodium falciparum* Lines *In Vitro*: Evidence for Decreased Sensitivity to Dormancy and Growth Inhibition

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The appearance of *Plasmodium falciparum* parasites with decreased *in vivo* sensitivity but no measurable *in vitro* resistance to artemisinin has raised the urgent need to characterize the artemisinin resistance phenotype. Changes in the temporary growth arrest (dormancy) profile of parasites may be one aspect of this phenotype. In this study, we investigated the link between dormancy and resistance, using artelinic acid (AL)-resistant parasites. Our results demonstrate that the AL resistance phenotype has (i) decreased sensitivity of mature-stage parasites, (ii) decreased sensitivity of the ring stage to the induction of dormancy, and (iii) a faster recovery from dormancy.

Artemisinin combination therapy (ACT) is the cornerstone of malaria control programs. However, reports of parasites in Cambodia with decreased *in vivo* sensitivity to artemisinin (ART) (4, 6) have raised serious concerns about the emergence of artemisinin resistance in the field. To gain insight into the mechanisms of ART resistance and to monitor its spread (5), it is critical to characterize the resistance phenotype. To date, it has not been well described, and no molecular marker has been identified.

Clinical data from western Cambodia indicated that parasite clearance times after artesunate monotherapy were significantly longer than those in northwestern Thailand (4). However, few other phenotypic changes were identified. The Thai and Cambodian isolates showed no significant differences in susceptibility (50% inhibitory concentrations [IC_{50}s]) to either dihydroartemisinin (DHA) or artesunate in classic *in vitro* drug susceptibility tests (4).

Reduced susceptibility of ring-stage parasites to ART has been proposed as an explanation for the prolonged clearance times observed in Cambodia (4, 7). This hypothesis would explain the results of conventional *in vitro* susceptibility tests, which assess the effect of a drug on the maturation of parasites from the ring to the schizont stage (4), and may be supported by an *in vitro* study showing that only ring-stage artemisinin-adapted parasites persisted during 48 h of drug pressure (10). In contrast, it has been reported that IC_{50}s for three laboratory parasite lines adapted to tolerate 80 ng/ml of artelinic acid (AL) increased 2- to 5-fold (1). These changes were accompanied by increases in copy number, mRNA expression, and protein expression of the *pfmdr1* gene. Using one of these adapted parasite lines, increases in IC_{50} of up to 12-fold after renewed drug selection were reported (2). The withdrawal of drug pressure resulted in deamplification of the *pfmdr1*-containing amplicon and in partial reversal of resistance to AL.

Another feature of *Plasmodium falciparum* parasites is their ability to enter a quiescent state (dormancy) following exposure to ART, where development of ring-stage parasites is temporarily arrested. ART-sensitive parasites can stay dormant for up to 20 days after a single exposure to DHA *in vitro*, with 0.044% to 1.313% of parasites recovering, depending on the drug dose and parasite strain (8). This phenomenon could explain the high frequency of recurrences in the field (3, 9). Changes in dormancy patterns may also be involved in ART resistance and may explain the discrepancy between the clinical response phenotype and *in vitro* susceptibility results of field isolates. For instance, it has been reported that dormancy recovery appears to be more rapid for *in vitro* drug-selected parasites than for sensitive parasites (10).

Here we describe the phenotype of AL-resistant *P. falciparum* laboratory lines for which a decrease in parasite susceptibility has been shown previously (1, 2). We compared the dormancy properties of these AL-adapted parasites and their sensitive parent lines post-drug treatment at a range of different concentrations. Moreover, we monitored the growth characteristics of parasites under continuous drug pressure.

MATERIALS AND METHODS

Preparation of parasites *in vitro*. AL-sensitive *P. falciparum* lines W2, D6, and TM91C235 were cultivated as previously described (8). Frozen stocks of AL-resistant lines W2AL80, D6AL80, and TM91C235AL80 (1) were thawed and reexposed to several cycles of treatment with 80 ng/ml AL (2 to 5 continuous days per round). Before the start of each experiment, parasites were synchronized at the ring stage by two rounds of sorbitol treatment.

Comparing the effects of AL on the growth of sensitive and resistant parasite lines. Ring-stage parasites of AL-sensitive and -resistant lines were placed into two preprepared drug-filled 96-well plates to a final volume of 200 μl (2% parasitemia; 3% hematocrit). The plates contained AL at doubling dilutions from 10 to 640 ng/ml. One plate was harvested after 30 h of incubation, and thin and thick smears were made from each well. Giemsa-stained thin smears were examined by light microscopy to assess the proportions of parasites at various developmental stages. The second plate was incubated for 24 h before the wells were washed with fresh medium and the drug was reapplied and incubated for a further 48 h.

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After this 72-hour exposure, the plate was washed, resuspended in drug-free medium, and incubated for an additional 48 h. After further washes, cultures were diluted 1:20 in fresh medium, and parasite growth in each well was quantified by measuring the amount of cellular HRP2, using an SD malaria antigen *P.f* enzyme-linked immunosorbent assay (ELISA) kit (Standard Diagnostics Inc., South Korea) following the manufacturer's instructions. Drug-free cultures were run in parallel to all experiments as positive controls. We defined the growth inhibition threshold as the lowest concentration which caused a >50% reduction in parasite growth after 72 h of continuous drug pressure relative to the positive control.

Comparing the induction of and recovery from dormancy of AL-sensitive (W2) and -resistant (W2AL80) parasites. Twenty-milliliter cultures of ring-stage parasites with an initial parasitemia of 2% were exposed to a range of AL concentrations (for W2, 640 to 5,120 ng/ml; and for W2AL80, 2,560 to 10,240 ng/ml) for 6 h, after which cultures were washed with fresh medium and returned to the incubator. Cultures were passed through a magnetic column (25 MACS CS separation column; Miltenyi Biotec) on 3 consecutive days, starting 24 h after treatment (8), to remove mature parasites (possibly unaffected by the drug). Cultures were then maintained until parasitemias reached ~10%. Sham-treated parasites (without drug and magnetic column treatment) were used as a control. Blood films were made daily, stained with Giemsa stain, and examined by light microscopy to determine parasitemia. The presence of dormant ring-stage parasites was also examined at 24 h posttreatment.

RESULTS

AL-resistant lines grow and multiply at higher concentrations of AL than sensitive lines. Based on ELISA results, the AL growth inhibition thresholds for the sensitive lines were 10 ng/ml for W2 and 40 ng/ml for D6 and TM91C235. For the resistant lines, the growth inhibition threshold was 2-fold higher for TM91C235AL80 (80 ng/ml), 4-fold higher for D6AL80 (160 ng/ml), and 16-fold higher for W2AL80 (160 ng/ml). These thresholds were consistent when a biological replicate was conducted.

Examination of blood smears taken after 30 h of drug exposure showed a dramatic difference in parasite maturation under drug pressure (Fig. 1A and B). For the resistant line W2AL80, more than 80% of the initial ring-stage parasites matured to the trophozoite or schizont stage when exposed to 40 ng/ml or less of AL (Fig. 1A), and 640 ng/ml of AL was required to almost completely stop parasite maturation. In contrast, fewer than 20% of AL-sensitive ring-stage parasites progressed to mature stages when exposed to only 10 ng/ml of AL; development was completely halted at AL concentrations above 10 ng/ml (Fig. 1A). The same trend was observed for the D6 and TM91C235 parent and resistant lines (data not shown). The quantity of HRP2 in W2AL80 after the 72-h drug incubation was 2.9- to 5.8-fold higher than in its sensitive parent at drug concentrations of ≤40 ng/ml (data not shown). In contrast, the ratio of HRP2 quantity in W2AL80 at the highest drug concentration (640 ng/ml) and in W2 in the no-drug control were 1.02 and 1.04. Combined, these results demonstrate that the AL-resistant parasite lines were able to mature and multiply in the presence of AL.

AL-resistant W2AL80 requires a higher drug concentration to induce dormancy than AL-sensitive W2. Following 30 h of incubation under drug pressure at concentrations ranging from 10 to 640 ng/ml AL, the proportions of dormant and dead rings among W2 and W2AL80 parasites were compared. For the purposes of this study, the dormant and dead ring-stage parasites were grouped together and are referred to as dormant henceforth because they could not always be differentiated by means of morphology. In W2 samples, 48% of parasites were dormant ring-stage parasites following 10 ng/ml exposure to AL. This figure increased to >86% at AL concentrations of 20 ng/ml and above (Fig. 1A). In contrast, only 45% of parasites were dormant rings in W2AL80 samples at 320 ng/ml of AL, a 32-fold increase in drug concentration compared to that with W2. At AL concentrations of <40 ng/ml, only a small proportion of dormant rings (≤4.3%) were observed in the resistant line (Fig. 1A).

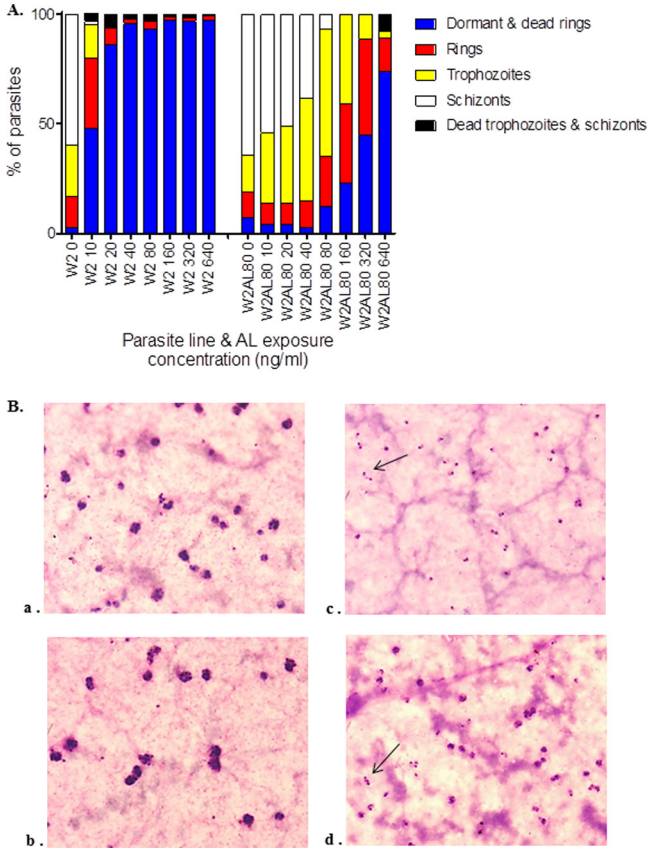

FIG 1 Proportions and morphologies of various parasite developmental stages after 30 h of continuous AL exposure. (A) Proportions of parasites at various developmental stages after 30 h of continuous AL exposure at concentrations ranging from 10 to 640 ng/ml for W2 and W2AL80. Cultures are labeled by parasite line followed by drug concentration. (B) Light microscopic pictures (thick smears) of W2 and W2AL80 parasites 30 h after continuous treatment with 80 ng/ml AL. (a) W2 control; (b) W2AL80 control; (c) W2 posttreatment; (d) W2AL80 posttreatment. Arrows point to typical dormant parasites.

W2AL80 recovers from dormancy earlier than W2. To assess how W2AL80 and W2 parasites recover after a 6-h exposure to AL, parasite cultures were monitored daily from day 4. Recovery data from days 1 to 3 are not available because the cultures were passed through a magnetic column on these days to ensure that growing parasites, which were possibly unaffected by the drug, were removed. Dormant ring-stage parasites could be observed on microscope slides at 24 h posttreatment at all drug concentrations. The AL concentrations used in this experiment were 64 to 512 and 16 to 64 times higher than the growth inhibition thresholds determined above from the 72-h exposure period for W2 and W2AL80, respectively. In the W2 cultures exposed to 640, 2,560, and 5,120 ng/ml AL, parasitemia remained below 1% until day 8. After day 8, parasitemia started to increase, with >10% parasitemia reached

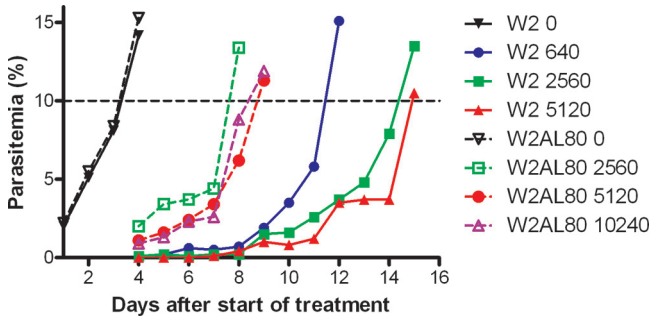

FIG 2 Dormancy recovery profiles of W2 and W2AL80 bulk cultures after treatment with AL at various concentrations (ng/ml) for 6 h, with passage through a magnetic column on days 1 to 3. Cultures are labeled according to parasite line and AL exposure concentration.

on day 12 for the 640-ng/ml AL-exposed cultures and on day 15 for the 2,560- and 5,120-ng/ml AL-exposed cultures (Fig. 2). In contrast, the W2AL80 cultures reached levels of >10% on day 8 after exposure to 2,560 ng/ml AL and on day 9 after AL exposures of 5,120 ng/ml and 10,240 ng/ml. Hence, all cultures recovered following exposure to ≥640 ng/ml AL, with W2AL80 recovering faster than W2 for a comparable effective drug concentration (relative to the growth inhibition threshold). The sham-treated cultures for both W2 and W2AL80 reached 10% parasitemia within 4 days (Fig. 2).

DISCUSSION

Parasites with decreased *in vivo* sensitivity to artemisinin have been reported, but *in vitro* these isolates did not appear significantly more resistant than those obtained elsewhere (4). The aim of this study was to define the resistance phenotype by characterizing the growth and dormancy recovery profiles of well-developed and -characterized AL-resistant parasite lines compared with their drug-sensitive parents. We used AL-resistant lines because these were the only available lines that had been well demonstrated to be resistant to ART derivatives and because there is a close structural relationship between AL and other ART derivatives, so similar phenotypes can be expected. *P. falciparum* parasite lines W2AL80, D6AL80, and TM91C235, used in this study, had been adapted to and were able to grow for at least 20 days under a drug pressure of 80 ng/ml AL. These parasites had shown relative increases in AL IC_{50} of up to 12-fold, suggesting true resistance (1, 2). Although these laboratory resistant lines may not be able to reflect all characteristics of the wild-type isolates, investigating their characteristics provides insights into the possible phenotypic and genotypic changes of ART-resistant parasites in the field.

In this study, at least 160 ng/ml of AL for 72 h was required to reduce parasite growth by 50% in the resistant line W2AL80. Examination of blood smears taken after 30 h under the same experimental conditions confirmed parasite maturation into schizont stages at concentrations up to 80 ng/ml AL, demonstrating not only that ring-stage parasites were able to develop into mature stages but also that trophozoites were able to develop through to schizonts. This contrasts with the results of Witkowski et al. (10), who observed no mature parasite stages during a 48-hour ART exposure of F32-ART, a laboratory line that had been adapted to survive 24-h drug cycles of up to 9 μM ART (~2,560 ng/ml). At this stage, it is not clear how our *in vitro* observations compare morphologically to parasites posttreatment in patients with adequate or long parasite clearance times.

A small proportion (≤12.3%) of dormant and dead ring-stage parasites could be observed in the resistant parasite line following 30 h of continuous exposure to the drug concentration to which the parasites had been adapted. However, this proportion was not significantly different from that of the sham-treated control samples. Only at concentrations above 80 ng/ml did this proportion increase, reaching ~45% at 320 ng/ml. This is in sharp contrast to the case for W2, where 48% of parasites were dormant at 10 ng/ml of AL. These results indicate that dormancy still occurs in the resistant parasite line but that higher drug concentrations are required to induce it. The majority of resistant parasites do not develop dormancy at concentrations they can tolerate. This also suggests that dormancy may be an innate mechanism that parasites use to survive drug concentrations which would otherwise cause severe damage and that it is triggered only when parasite development is significantly inhibited.

To monitor recovery from dormancy, we exposed W2 and W2AL80 to concentrations well above the IC_{50} for 6 h. At these high concentrations, dormant ring-stage parasites could be observed on microscope slides at 24 h posttreatment at all drug concentrations. The use of a magnetic column on 3 consecutive days ensured removal of any parasites that might not have gone into dormancy. AL-resistant parasites recovered from dormancy after treatment with concentrations as high as 10,240 ng/ml. Recovery occurred significantly earlier in W2AL80 than in W2 following exposure to a comparable effective drug concentration. These results are consistent with earlier findings which also showed delayed recovery in sensitive lines compared to ART-resistant lines *in vitro* (10).

In summary, AL-adapted parasites show resistance to AL at all developmental stages. Exposure to AL can induce dormancy in resistant ring-stage parasites, but much higher drug concentrations are required to induce this arrest. Recovery from dormancy occurs at a higher rate than that of the AL-sensitive parent line, although it is not possible from this study to determine if this is due to an earlier resumption of normal growth or a larger proportion of parasites recovering from dormancy.

These results indicate that ART-induced dormancy and resistance are linked in the 3 AL-resistant lines we studied. At this stage, it is not clear how these findings relate to the delayed clearance observed in field isolates. Based on our observations, we hypothesize that the first step toward the development of ART resistance is the development of resistance in the ring stages, which reduces the proportion of parasites entering a dormant state. The second stage of resistance is resistance in mature-stage parasites, which allows the parasites to survive and replicate in the presence of drug. Our AL-resistant parasites have been developed over years of repeated drug exposure, so they are likely to be at a more advanced stage of resistance than the field isolates collected in Cambodia. Therefore, decreased sensitivity in both ring stages, i.e., changes in dormancy pattern, and mature stages is prominent in these laboratory lines. In contrast, field isolates may be in the first stage of resistance, where only changes in the sensitivity of ring stages have developed, which enables them to bypass dormancy and develop into trophozoites. Since isolates have not entered the second stage of resistance, trophozoites are still sensitive to and are killed by ART. This may explain the delayed parasite clearance following treatment. Further studies using field isolates are needed to evaluate this

hypothesis. Understanding the phenotypic changes which occurred in these resistant parasites will contribute to a better understanding of the resistance mechanisms involved so we can develop tools to monitor for and contain ART resistance.

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