THE CHANGING IN VITRO SUSCEPTIBILITY PATTERN TO PYRIMETHAMINE/SULFADOXINE IN PLASMODIUM FALCIPARUM FIELD ISOLATES FROM KILIFI, KENYA

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Abstract. Two clinical trials that used Falcidin® (Cosmos Ltd., Nairobi, Kenya), the antifolate combination of pyrimethamine/sulfadoxine (PM/SD), as treatment for non-severe falciparum malaria in children at Kilifi, Kenya in 1987–1988 and 1993–1995 have presented an opportunity to assess in vitro the susceptibility trend of Plasmodium falciparum to PM and SD over time on the Kenya coast. The first set of isolates was collected prior to the introduction of PM/SD into the Kenya Medical Research Institute/Wellcome Trust Research unit while the second set was taken soon after PM/SD was introduced in the study area as the first-line treatment drug for uncomplicated falciparum malaria. In the first trial, 69 isolates collected before and after treatment of malaria with PM/SD were tested directly in the field for susceptibility to PM and SD using the standard in vitro micro-test technique, with minimal levels of folate. In the second trial, 97 isolates similarly collected were adapted to culture, and tested as described elsewhere. In both studies, PM and SD susceptibility tests were done separately. There was a highly significant decrease (P < 0.01) in the in vitro sensitivity of P. falciparum isolates to PM and SD between the two trials. In the first trial, the isolates were either sensitive to both PM and SD or resistant to PM and sensitive to SD. During the second trial, isolates were either resistant to PM and sensitive to SD or resistant to both drugs. These results are important in estimating the useful therapeutic life (UTL) of PM/SD in this area and in identifying alternative antifolate drugs.

INTRODUCTION

In Southeast Asia and South America, the extensive use of pyrimethamine/sulfadoxine (PM/SD)-selected Plasmodium falciparum populations resistant to this drug very rapidly. As a result, PM/SD is being used increasingly as the first-choice treatment for chloroquine-resistant falciparum malaria, despite evidence that this combination is already emerging within the East African sub-region. Few studies have addressed the changes in drug sensitivity within a specific area during the early stages of drug use, which may provide early warning signs of drug resistance. The mechanism of clinical failure after treatment with PM/SD is not well understood. Resistance to PM and SD has been separately documented, and it is now clear that the mechanism of resistance involves point mutations in the two target genes, dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS). Although emerging data support the conclusion that resistant isolates carry point mutations in both the enzymes targeted, the strong synergy exhibited by PM and SD may make it difficult to accurately define the forces responsible for the selection pressure. The three processes, the mode of action, synergy, and resistance mechanism, are interconnected and each must be clearly understood. This makes the definition of the individual components and their interaction more complex. The rapid selection and spread of P. falciparum isolates that demonstrate reduced sensitivity to PM and SD are of great concern in malaria-endemic regions of sub-Saharan Africa, since there are no available and affordable alternative drugs. In Kilifi, Kenya, an area of continuous malaria transmission, the routine use of this drug combination started in the early 1990s, and involved more than one source. The main source was the Kenya Medical Research Institute/Wellcome Trust Research Unit, which started using this combination as the first choice treatment for non-severe falciparum malaria in 1992. The outpatient department of the district hospital recommended the use of PM/SD plus chloroquine from 1990; however, this recommendation was subject to the availability of PM/SD (Marsh K, unpublished data).

Previous community surveys in this area showed that the only anti-malarial drug available in the local shops is chloroquine. The role of private clinics in the supply of PM/SD seems to be fairly minimal since only 2–5% of mothers from this area seek treatment for their children from such sources. Currently, there is practically no PM/SD in the local shops (Marsh V, unpublished data). The increase in routine use of this drug combination occurred despite previous in vitro data suggesting that this combination rapidly selects for PM-resistant isolates in this area. Clinicians working in the unit had no choice other than use of PM/SD, since all other alternative drugs were too expensive. In this unit, either Fansidar® (pyrimethamine, 25 mg; sulfadoxine, 500 mg; F. Hoffman-LaRoche, Basel, Switzerland) or Falcidon® (same content; Cosmos Ltd., Nairobi, Kenya) is used depending on availability. The two preparations have comparable bioavailability in healthy Kenyan adults. The primary goal of our study was to investigate the transition from PM/SD sensitivity to resistance in P. falciparum field isolates from Kilifi, Kenya by analyzing changes in the in vitro susceptibility to PM and SD of parasite isolates obtained over a period of increasing use of PM/SD.

MATERIALS AND METHODS

The study population for both studies was composed of children between 6 and 71 months of age. Febrile children,
FIGURE 1. Correlation between pyrimethamine (PM) and sulfadoxine (SD) in vitro susceptibility for 1987–1988 pretreatment (□) and post-treatment (×) isolates. Pre denotes pretreatment and Post denotes post-treatment isolates. Quadrants: A = SD S + PM S, B = SD S + PM R, C = SD R + PM R, D = SD R + PM S. The number of both infection types that fell within each quadrant is indicated.

The study area in Kiliﬁ District, Kenya has an average entomologic inoculation rate (EIR) of four infective bites per year (range 0–18), an annual EIR of 60 has been recorded for a site with the highest transmission intensity. This area is also characterized by continuous transmission with pronounced seasonal variation and relatively high incidence of severe disease occurring in time-space clusters, and patients frequently carry polyclonal isolates. This phenomenon is consistent with naturally occurring infections found in other areas.

The in vitro chemosensitivity test methods for both trials were comparable in two main aspects; they both used minimal folate medium and provided at least 48 hr of parasite-drug contact. Both studies collected pretreatment and post-treatment parasite samples either by active follow-up or whenever the study patients returned to the clinic. This sampling method sought to investigate in vitro chemosensitivity differences resulting from treatments in addition to determining the interval between episodes. Samples isolated from patients treated with PM/SD in 1987–1988 were tested for sensitivity to PM and SD and used to establish a baseline. In this analysis, we also compared the mean drug concentration producing 50% parasite inhibition (IC50) value for PM and SD in both trials. The in vitro susceptibility of 1993–1995 isolates to other antifolates and their DHFR genotypes have been published elsewhere. The Epi-Info version 6 (Centers for Disease Control and Prevention, Atlanta, GA.) package was used for statistical analyses, testing significance at P < 0.05. Graphic presentation was done using Unistat-IV (Magelon, London, United Kingdom) and Microsoft Excel® (Microsoft Corporation, Redmond, WA). The Kenya National Ethical Review committee approved both trials, and informed consent was obtained from parents or legal guardians of children who took part.

RESULTS

Figures 1 and 2 show data for the 1987–1988 and 1993–1995 isolates, respectively. The sensitivity of each isolate to PM and SD is plotted on a log scale, and the figures are divided into four quadrants. To define sensitivity (S) and resistance (R) to PM and SD, we used IC50 values based on previous in vitro susceptibility for reference isolates.
lates with an SD $IC_{50}$ > 10 $\mu$M were defined as SD resistant (SD$^R$) and those with a PM $IC_{50}$ > 10 nM were defined as PM resistant (PM$^R$). Isolates with lower $IC_{50}$ values were SD sensitive (SD$^S$) and PM sensitive (PM$^S$), respectively. Each isolate was designated to one of four quadrants: PM$^S$ + SD$^R$ (quadrant A), PM$^R$ + SD$^R$ (quadrant B), PM$^R$ + SD$^S$ (quadrant C), and PM$^S$ + SD$^S$ (quadrant D). Using the above classification, 28 (41%) of 69 isolates from the 1987–1988 trial were PM$^R$. However, in this group only 2 isolates were SD$^R$, and one showed borderline sensitivity to both drugs (Figure 1).

The isolates from the 1993–1995 trial showed a very different pattern of susceptibility (Figure 2). There was a significant increase in the $IC_{50}$ values for both drugs ($P < 0.01$) and 65 (67%) of the 97 isolates fell within quadrant B that represents PM$^R$ SD$^R$. However, in this group only 2 isolates were SD$^R$, and one showed borderline sensitivity to both drugs (Figure 1).

The isolates from the 1993–1995 trial showed a very different pattern of susceptibility (Figure 2). There was a significant increase in the $IC_{50}$ values for both drugs ($P < 0.01$) and 65 (67%) of the 97 isolates fell within quadrant B that represents PM$^R$ SD$^R$. The 1993–1995 pretreatment parasite populations were a mixture of isolates that spanned the range from sensitivity to intermediate resistance or resistance to PM and SD (Figure 2). The post-treatment samples in both trials were composed of two distinct populations of sensitive and resistant isolates. However, a comparison between pretreatment and post-treatment samples from in the 1993–1995 trial showed a reduction of isolates in the intermediate range of susceptibility. The mean $IC_{50}$ values for PM and SD against 1993–95 pretreatment and post-treatment isolates were not significantly different ($P = 0.098$ and 0.103, respectively). A comparable but less pronounced trend was observed for the 1987–1988 samples, which had relatively fewer pretreatment isolates.

Not all patterns of resistance were observed. Even in the 1987–1988 study, well before PM/SD was available for routine use in this area, we found some isolates that were resistant to PM but still sensitive to SD (Figure 1, quadrant C). Apart from two isolates derived from the 1993–1995 study, all others that showed resistance to SD were resistant to PM as well (Figure 2, quadrant B). Only two isolates from the 1987–1988 study were SD$^R$ + PM$^S$ (Figure 1, quadrant A) and one was borderline SD$^R$ + PM$^S$ (Figure 1, quadrant B). Sixty-six (96%) of 69 of the 1987–1988 isolates were confined to quadrants C and D of Figure 1, distributed in the ratio of 1:1.4, respectively. In contrast, 85 (88%) of the 97 isolates from the 1993–1995 trial were confined to quadrant B and C of Figure 2, in the ratio of 1:0.31, respectively.

The in vitro susceptibility data for both trials are shown in Table 1. There was a 10-fold increase in $IC_{50}$ values for both drugs between the two study periods. In terms of PM susceptibility, the proportion of PM$^S$ decreased from 59% to...
was unique in two ways. The presence of PM resistant (PM R) isolates in the 1987–1988 study was rare, but that the force(s) responsible for the selection and maintenance of PM R isolates in the 1987–1988 isolates was sensitive to SD, whereas the corresponding value for 1993–1995 isolates was 30%. The clinical correlates of the 1987–1988 study were as described. Briefly, parasites reappeared only after day 35 following treatment; as expected, the PM/SD afforded a substantial period of chemoprophylaxis. The 1993–1995 clinical outcomes have also been reported in detail elsewhere. At this later time, there was a clear decrease in the parasitologic response to PM/SD since 10% of the patients were still parasitemic on day 7 following treatment. This was a clear indication of the existence of a strong selective pressure in individual patients. The presence of three (21.5%) of 24 PM R isolates in the 1987–1988 samples, even before the introduction of PM/SD for routine use (Figure 1, quadrant C). Twenty-four (89%) of 27 isolates in quadrant C in Figure 1 were from post-treatment samples, a clear manifestation of the existence of a strong selective pressure in individual patients. The presence of three (2.15%) of 24 PM R isolates in the 1987–1988 pretreatment samples indicated the occurrence of PM R alleles prior to PM/SD use. Such alleles could also be maintained in P. falciparum populations at very low frequencies by the use of common antibacterial agents, such as trimethoprim/sulfamethoxazole. This drug combination also acts to inhibit folate biosynthesis and use, but since it is characterized by weak anti-malarial activity and a short elimination profile, it may exert a weak antifolate resistance selection pressure in P. falciparum. This might be the case among patients treated with trimethoprim/sulfamethoxazole for bacterial infections, while simultaneously harboring malaria parasites, although there are some data to the contrary.

The second, and the most interesting finding was the increase in IC50 values that was mainly observed on the background of PM R and not among PM S isolates. This was clearly demonstrated by the fact that only four isolates (two from each trial) were SD resistant (SD R) + PM R. The absence of SD R + PM R isolates (Figure 1, quadrant B) in the 1987–1988 samples implied that this phenotype was not only rare, but that the force(s) responsible for the selection and maintenance of PM R was/were not as effective for the simultaneous selection of SD R at that time. The change in IC50 values for the field isolates against PM and SD was in keeping with those observed in P. falciparum reference isolates whose DHFR and DHPS genotypes are known.

The 1993–1995 isolates have shown that within a period of six years, during which PM/SD was increasingly used for routine treatment of non-severe falciparum malaria, there was a significant change in the in vitro susceptibility of P. falciparum to PM and SD in the study area. Additionally, there was a substantial reduction of isolates with intermediate susceptibility to PM since parasite resistance to PM and SD increased, especially in the post-treatment infections. The comparison between 1993–1995 pretreatment and post-treatment samples demonstrated the existence of a mild selective pressure exerted by PM/SD treatment in individual patients, in contrast to a previous observation in this area. During the 1993–1995 study, the within-patient selection was less dramatic and seems to have occurred against isolates with an intermediate range of susceptibility to PM. The rapid increase in PM and SD IC50 values observed after PM/SD was introduced in this area reflects the ease with which selection for higher levels of resistance to this drug combination progresses. These data are of great concern and call for urgent research into the introduction of effective, safe, and affordable alternatives to PM/SD for routine use. Our group has identified and extensively studied a potential antifolate drug combination that may fit these criteria.

Although the adaptation of parasites to in vitro culture may reduce the diversity of the samples, the short-term cultures used in this study (mean ± SD: 48.66 ± 29.25 days) were unlikely to have selected one or a few parasites at the expense of others, especially in terms of their PM susceptibility. Additionally, our previous report showed that 60% of the adapted samples were mixed isolates since both wild and mutant alleles of dihydrofolate reductase (DHFR) was observed. The data from these trials support previous findings suggesting that the prevalence of in vitro PM R is correlated with increased use of and resistance to PM/SD. Both studies have also provided more information on the relationship between the in vitro susceptibility of P. falciparum to PM and SD as a function of PM/SD use. Our data suggest that the change in PM and SD in vitro activities (and by extension, the change in DHFR and dihydropteroate synthase [DHPS] genotypes) occurred at the same rate between the two trials.

The molecular bases of P. falciparum in vitro resistance to PM and SD have been described; these are mutations in the DHFR and DHPS genes, respectively. It is therefore possible that the observed increase in SD IC50 values for 1993–1995 isolates was a function of DHPS mutations, since the primary target for SD and other sulfonamides/sulfones when acting alone is DHPS, especially under low or zero folate levels. Our data indicate that the critical factors that lead to in vitro PM R and SD R are increased use of PM/SD, which selects for and accumulates mutations in the DHFR domain (especially triple mutants). When PM/SD use is continued in populations that are comprised principally of parasites that carry the triple mutant allele of DHFR, DHPS mutants are also selected. The result is a population of parasites that carries alleles of both DHFR and DHPS that confer high levels of resistance to PM/SD. Our group has DHFR and DHPS genotype data supporting this hypothesis (Nezila AM, unpublished data). A possible explanation for the observed asymmetry in the selection pattern between DHFR and DHPS could be that as long as PM is still effective, the selective pressure on DHPS is minimal. However, when mutations on the DHFR gene accumulate and reverse PM activity, SD becomes the only effective drug being adminis-
tered. Under such conditions, there is selective pressure on the DHPS domain, and the observed increase in SD IC₅₀ on PM⁶ isolates would be the expected outcome. Recent genotyping data suggest that PM/SD treatments select for mutations on the DHFR and DHPS domains simultaneously as observed in isolates from Iquitos, Peru.¹⁵ However, these data are derived from an epidemic situation likely to originate from a limited number of sources. The genetic diversity in parasites isolated from such infections would clearly reflect limited population characteristics and the same pattern of selection may not be common in areas of continuous malaria transmission such as Kilifi.

The selection pattern observed in this area was in keeping with the hypothesis of simultaneous and synergistic inhibition on DHFR by PM and SD⁴⁰–⁴¹ and supports the proposed model for PM/SD resistance development in Africa.¹⁶ This model has since been criticized⁶² and more arguments for and against it put forward.⁴³–⁴⁴ The general trend from these two trials demonstrated an initial and consistent increase in PM⁶ that occurred prior to the appearance of SD⁸. The only exception to this trend has been reported in Mali, an area with limited use of PM/SD, suggesting that the selection for DHPS mutations may have been due to the use of sulfadrug(s) alone in that setting.¹⁴ Additionally, it is possible that the selection of SD⁶ field isolates reported here may be a function of the observed 3-fold difference in SD sensitivity between SD³ and SD⁶ reference isolates in physiologic folate medium.¹⁶ This difference, although small, may be sufficient to select isolates with reduced SD sensitivity.

Finally, our data suggest that in vitro chemosensitivity tests, although uneconomical for large-scale screening, can be used for monitoring the efficacy of other antifolate anti-malarials in the absence of molecular biotechnology facilities and may be useful for drugs whose molecular bases of resistance are yet to be defined. The present work has also underscored the importance of periodic monitoring of anti-malarial drug efficacy, based on parasite in vitro and in vivo susceptibility on which rational treatment policies can be derived. A crucial part of this exercise should include the definition of drug resistance levels (both parasitologic and clinical) that would form the basis for the replacement of anti-malarial compounds that are in routine use, in addition to ensuring that there are appropriate alternatives.

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