Sulfadoxine-Pyrimethamine Resistance in the Rodent Malaria Parasite Plasmodium chabaudi

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Received 1 April 2002/Returned for modification 25 April 2002/Accepted 9 May 2002

We have studied resistance to sulfadoxine-pyrimethamine (S/P) in the rodent malaria parasite Plasmodium chabaudi. A stable S/P-resistant mutant, AS(50S/P), was selected by drug treatment of a clone, AS(PYR), already resistant to pyrimethamine. The sequences of the P. chabaudi dhfr and dhps genes were obtained and found to be identical in AS(50S/P) and AS(PYR), showing that resistance to S/P in AS(50S/P) was not due to additional mutations in either gene. AS(50S/P) was crossed with a drug-sensitive clone, AJ, and 16 independent recombinant progeny were obtained. These clones were phenotyped for their susceptibility to S/P and to sulfadoxine and pyrimethamine separately. Pyrimethamine resistance was invariably associated with S/P resistance, but no correlation was found between resistance to S/P and resistance to sulfadoxine. Quantitative trait locus analysis of the progeny with 31 chromosome-specific markers showed that resistant P. chabaudi dhfr, or one or more genes closely linked to it, was a major determinant of S/P resistance. In addition, the inheritance of genes on chromosomes 5 and 13 from the sensitive parent appeared to contribute to the level of resistance observed. These results demonstrate that the S/P resistance of the AS(50S/P) mutant of P. chabaudi does not involve mutation in dhps and is not due simply to a combination of two genes determining resistance to pyrimethamine and sulfadoxine separately.

The spread of chloroquine resistance in Plasmodium falciparum has favored the combination of sulfadoxine and pyrimethamine (S/P) (Fansidar) as one of the most important and widely used treatments for malaria (3). However, S/P resistance is now widespread in Southeast Asia and South America and is spreading through Africa (44). While the genetic mechanisms involved in resistance to pyrimethamine are well understood, only circumstantial evidence on the genetic basis of S/P resistance is available.

Pyrimethamine is an antifolate drug which inhibits dihydrofolate reductase (DHFR), an essential enzyme in the parasite’s folic acid pathway. Sulfadoxine, a sulfa drug, is an analogue of p-aminobenzoic acid (PABA) and competitively inhibits dihydropteroate synthase (DHPS), which also is required for folate biosynthesis. In combination, these drugs exhibit a high level of synergy (10). In P. falciparum, pyrimethamine resistance is clearly due to amino acid changes in DHFR, in particular a change at position 108 of serine (Ser) or threonine (Thr) to asparagine (Asn) (27, 30, 45). Similarly, genetic and transfection studies have shown that sulfadoxine resistance in vitro is associated with mutations in P. falciparum dhps (4, 33–35, 40).

No experimental work has been carried out on the genetics of S/P resistance. In general, it is assumed that parasites exhibiting S/P resistance contain mutations in both P. falciparum dhfr and dhps, although some studies suggest that S/P failure may be determined simply by several mutations in dhfr alone (2, 22, 43). A major problem in studying S/P resistance in P. falciparum is the lack of a reliable in vitro test for phenotyping parasite responses to the S/P combination, due to variations in levels of the antagonists PABA and folate in serum and erythrocytes used for routine culture (21, 41, 42). Therefore, the identification of alleles associated with S/P resistance in natural populations has been based only on molecular assays to detect genotypes associated with therapeutic failure (15, 29, 39). However, predictions of the outcome of S/P treatment based on mutations in P. falciparum dhfr and dhps have had only limited success (15, 22, 23, 28).

We report here a genetic study on S/P resistance using the rodent malaria species Plasmodium chabaudi, which is probably the best rodent model for studies on drug resistance in P. falciparum (7). Pyrimethamine resistance in P. chabaudi is known to be associated with the presence of Asn-106 in its DHFR, which is equivalent to Asn-108 in P. falciparum DHFR (9). We first describe the selection of a mutant exhibiting stable S/P resistance, starting from a P. chabaudi clone already resistant to pyrimethamine. We then report the isolation and sequence of the P. chabaudi pppk-dhps gene, which encodes the bifunctional protein hydroxymethylpterin pyrophosphokinase (PPPK)-DHPS, as well as those of the P. chabaudi dhfr-ts gene, encoding DHFR-thymidylate synthase, from the S/P-sensitive and -resistant clones. No additional mutations are seen in either gene following the development of S/P resistance. Genetic crossing work shows that S/P resistance is linked to pyrimethamine resistance but is independent of resistance to sulfadoxine alone. Quantitative trait locus (QTL) analysis of the cross progeny shows that P. chabaudi dhfr on chromosome 7 and genes on chromosomes 5 and 13 are strongly linked to S/P resistance and that another gene determining resistance may be located on chromosome 4.

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Materials and Methods

Parasites and hosts. The parasites used in this work were cloned lines of *P. chabaudi* isolates AS and AJ originating from independent wild-caught *Thomomys rutilans* thicket rats from the Central African Republic (8). Clone AS(PPYR) is pyrimethamine resistant and was selected from a sensitive AS clone designated AS(SENS) (38). Clone AJ is a wild-type parasite which is sensitive to pyrimethamine and innately resistant to sulfadoxine, as seen in all other isolates of this species so far examined. Parasites were maintained essentially as described previously (37), using adult CBA/Ca mice (University of Edinburgh, Edinburgh, United Kingdom, or Bantim and Kingman, Hull, United Kingdom) fed on SDS Formula Number 1 diet (Special Diets Services Ltd., Essex, United Kingdom), with the drinking water supplemented with 0.05% PABA to enhance parasite growth (14). Animal procedures were conducted under license following the United Kingdom Animals Scientific Procedures.

Drugs. Pyrimethamine base was obtained from Wellcome Research Laboratories (Kent, United Kingdom). Sulfadoxine and Fansidar were donated by Roche Pharmaceuticals. Drugs were made up as solutions in dimethyl sulfoxide (Sigma, Poole, United Kingdom) and administered orally to mice.

Selection for S/P resistance. One hundred outbred mice (Bantim and Kingman) were each inoculated intraperitoneally (i.p.) with 106 AS(PPYR)-infected red blood cells on day 0. A high curative dose of S/P (125/5.25 mg kg−1) was administered to each mouse daily from day 3 for a total of 4 days. Blood smears were then examined every 2 days from day 10. Blood smears were taken from any mice exhibiting parasitemia and stored as stabiles in liquid nitrogen. Lines chosen for further studies were cloned by limiting dilution (38). One of these clones, designated AS(SENS), was selected by the manufacturer's instructions (38).

Tests for drug resistance. A total of 104 parasitized red blood cells were inoculated i.p. into groups of five 6- to 9-week-old CBA/Ca inbred mice (Bantim and Kingman). Dilutions of pyrimethamine, sulfadoxine, or S/P were administered to three of the mice in each group at 3 h after inoculation and at the same time of day for the next 3 days. The remaining two mice in each group were undrugged controls. Drug doses were expressed as milligram per kilogram of mouse body weight. Blood smears were taken from day 6 onwards, and parasitemias were counted microscopically. Each parasite clone was tested for responses to each drug at least twice.

Stability of resistance in the absence of drug. Parasites were examined for the stability of their drug response by weekly passage through mice without drug pressure. Each week, the parasites were also subpassaged into further groups of mice and tested for their responses to pyrimethamine, sulfadoxine, and S/P. Their resistance was also tested following transmission through mosquitoes (*Anopheles stephensi*) (38).

Sequencing of the *P. chabaudi pppk-dhps* and *dhfr-ts* genes. To sequence the *P. chabaudi pppk-dhps* gene, known sequences of dhps (4, 12, 17, 24, 31, 33, 36) were aligned using the University of Wisconsin Genetics Computer Group DNA analysis software to identify two regions of sequence conservation. Degenerate oligonucleotides designed DHPS1 [TGTGTG(T/Y)(T/A)(T/C)AGATGG] and DHPS2 [GATTTGAC(T/C)(T/A)(T/C)(T/A)ATATC] were designed for use in PCR. Genomic DNA was isolated from AS(SENS) as described previously (32). A PCR product of approximately 100 bp was obtained and purified using Concert (Gibco BRL) according to the manufacturer's instructions and sequenced using BigDye (Perkin-Elmer) chemistry on an ABI 377 Prism Sequencer.

Sequences were extended to include the complete *P. chabaudi dhfr* and *pppk* genes by PCR walking using *P. chabaudi* Vectorset II (Genosys) libraries generated from genomic AS(SENS) DNA as described previously (1). Gene-specific primers were designed from the initial PCR product and used to generate additional sequence from the libraries, according to the manufacturer's instructions. The resulting sequence was used to design oligonucleotides (DHPS3, GAAAGACAAATATGTGCG; DHPS4, AATCTCATACCTAGTGATCC; DHPS5, TTTCCTGCAACATTGATCG; DHPS6, TATACGAGGTGTGATATG; PPPK1, GAAAAGCAATAAAATAAATACTACG; PPPK2, GAAAATCTTTAAAAACTCGGC; and PPPK3, CTCCGATAGTCTTAAATATGTAAG) to obtain the sequence of the *P. chabaudi pppk-dhps* gene from AS(PPYR), AS(SENS), S/P, and AJ, and each of the lines that survived S/P selection in order to detect any mutation events.

To sequence *P. chabaudi dhfr-ts*, fragments of the gene were amplified from parasite DNA by using primers designed from available sequence (11). The primers used to amplify the gene from genomic DNA of AS(SENS) and AS(SENS) were DHFR1 (GAAGATATCTCTGAAATATTCGATATATG), DHFR2 (TCTCTGATATCTGGAATAACATCAGC), DHFR4 (CAATGAAACTACATTATGTTCT), DHFR5 (GATTGTTGATGGCTTAATT)TGAATTTTAGAG), TS1 (AGAGAAGTTAATGACTTAGGTCC), and TS2 (TAAG CTGCCATATCCACTAG). Amplification products were purified and sequenced as described for *P. chabaudi pppk-dhps* above.

Crosses and recovery of progeny. In order to determine the genetic basis of resistance to S/P, AS(50S/P) was crossed with the unrelated drug-sensitive clone AJ (5). Two crosses were made using the basic procedure of Walliker et al. (38), by allowing mosquitoes (*A. stephensi*) to take up blood containing gametocytes of a mixed infection of the two clones, thus allowing cross-fertilization of gametes and recombination to occur. The resulting sporozoites were used to infect mice. The blood forms which developed in these animals, designated the progeny of the cross, were cloned by injecting inocula containing an average of 0.75 parasitized cell i.p. into mice (38).

Inheritance of chromosome-specific markers and QTL analysis. Chromosome markers developed by Carlson et al. (5) were used in linkage analysis of the cross progeny. Details of these markers can be found at http://www.ncbi.nlm.nih.gov/ Malaria/Rodent/ctabgenmarker.html. Details of marker 100 (pccg10) were provided by P. Hunt (personal communication). The inheritance pattern of each marker was compared with the inheritance of S/P, sulfadoxine, and pyrimethamine responses of each progeny clone. The data were analyzed by QTL methodology (16, 18) to determine whether inheritance patterns of the three types of resistance were consistent with a single or multigenic basis for each character. Since malaria parasites are haploid, this was simply carried out by calculating the mean difference in log parasitemias on day 6 following treatment with either 25/1.25 mg of S/P kg−1, 10 mg of pyrimethamine kg−1, or 150 mg of sulfadoxine kg−1 between groups of progeny clones inheriting either the AS(50S/P) or the AJ allele of each marker. The statistical significance was determined using Student's *t* test. The Holm procedure was used to correct genome-wide thresholds to account for the analysis of multiple chromosomal markers and drug resistance traits (40).

Nucleotide sequence accession number. A 2,489-bp sequence encoding a portion of the *P. chabaudi pppk-dhps* gene has been deposited in the DDBJ/EMBL/GenBank databases under accession number AJ302077.

Results

Selection for S/P resistance. An S/P-resistant mutant of *P. chabaudi* was selected by single-step exposure of a clone already resistant to pyrimethamine, AS(PPYR), to a high dose of the drug combination (125/5.25 mg of S/P kg−1) given over 4 days. Fifteen of 100 mice infected and treated with S/P developed patent parasitemias on day 18 after inoculation, 15 days after the last dose of S/P. The growth patterns of each of the S/P-selected lines were compared under S/P pressure to that of the parent clone AS(PPYR). Seven had drug responses comparable to that of AS(PPYR), while eight grew more quickly following treatment with 50/2.5 mg of S/P kg−1 (data not shown). These parasite lines were classified as S/P resistant.

One S/P-resistant line was cloned by limiting dilution and designated AS(50S/P). This clone was tested for its response to four daily doses of either (i) 25/1.25 mg of S/P kg−1, (ii) 10 mg of pyrimethamine alone kg−1, or (iii) 150 mg of sulfadoxine alone kg−1 (Fig. 1). Under S/P treatment, AS(50S/P) grew almost as well as parasites in undrugged mice; AS(PPYR) grew much more slowly, with recrudescence being observed only on day 10; and AS(SENS) failed to grow during the 10-day period (Fig. 1A). Under pyrimethamine treatment, AS(50S/P) grew slightly faster than AS(PPYR), while AS(SENS) was completely sensitive (Fig. 1B). Under sulfadoxine treatment, AS(50S/P) was slightly resistant, recrudescing on day 8 following drug treatment (Fig. 1C). In contrast, AS(PPYR) was completely eliminated by sulfadoxine, a result which was not unexpected given that selection for pyrimethamine resistance in rodent malaria parasites invariably results in an increased PABA requirement of the parasite and a consequent increased sensitivity to sulfa drugs (14). AS(SENS) was unaffected by sulfadoxine, and its growth was comparable to that observed in...
undrugged mice. The responses of AJ to all three drug regimens were comparable to those of AS(SENS).

Stability of resistance in the absence of drug. AS(50S/P) was passaged weekly for 20 weeks through uninfected mice without drug pressure, and each week parasites were tested for their drug responses. AS(50S/P) retained its original level of resistance to S/P, pyrimethamine alone, and sulfadoxine alone. It was also transmitted through A. stephensi into mice and was found to have retained identical responses.

Sequences of the \textit{P. chabaudi} \textit{pppk-dhps} and \textit{dhfr-ts} genes. Using degenerate oligonucleotides and Vectorette PCR (1), a 2,489-bp sequence which appeared to encode a portion of the \textit{P. chabaudi} \textit{pppk-dhps} gene was identified (data not shown). The sequence lacked approximately 10 amino acids at the amino terminus of the \textit{P. chabaudi} \textit{pppk}, based on comparison with the \textit{P. falciparum} gene.

The putative \textit{P. chabaudi} \textit{dhfr} gene was analyzed in all lines that survived drug selection, including AS(50S/P) (data not shown). Sequences from all of these lines were identical to that from AS(PYR). We also sequenced the \textit{pppk} portions of the \textit{P. chabaudi} \textit{pppk-dhps} genes from AS(PYR), AS(50S/P), and the drug-sensitive clone AJ. Again, all three sequences were identical. These results suggest strongly that resistance to the S/P combination is not due to mutations in \textit{P. chabaudi} \textit{pppk-dhps}. Although we were unable to obtain sequences for approximately 10 codons at the amino terminus of \textit{P. chabaudi} \textit{PPPK}, no polymorphisms have been reported in this region among alleles of \textit{P. falciparum} \textit{pppk-dhps}.

\textit{P. chabaudi} \textit{dhfr-ts} was sequenced from AS(SENS), AS(PYR), AS(50S/P), and AJ and compared to sequences previously published for \textit{P. chabaudi} (data not shown) (9, 11). In addition, \textit{P. chabaudi} \textit{dhfr} was sequenced from all the S/P-selected lines. The AS(SENS) \textit{dhfr} and the AJ \textit{dhfr} contained a Ser (AGT) at position 106. In contrast, as expected, the AS(PYR) \textit{dhfr} and AS(50S/P) \textit{dhfr} were identical and possessed Asn (AAT) at this position (9).

Cross results. Two crosses were carried out between AS(50S/P) and AJ. Thirty-three parasite lines were obtained in mice by limiting dilution of the progeny of the crosses, 14 from cross 1 and 19 from cross 2. Examination of these lines for 31 markers distinguishing each parent revealed that six contained two alleles of several parent clone markers and were thus mixtures of more than one clone. The remaining 27 lines were classified as pure clones by their possession of single alleles of all markers. Two of these were pure AJ parent type, one was pure AS parent type, and the remaining 24 clones were recombinants, exhibiting nonparental combinations of parent alleles. Eight of these clones had genotypes identical to that of at least one other recombinant clone and were therefore considered to be derived from duplicate cloning events. In total, therefore, 16 independent recombinants were identified, 8 from cross 1 and 8 from cross 2, and these were used in the subsequent linkage analysis work (Fig. 2).

Drug-responses of progeny clones. The growth rates of each progeny clone treated with S/P, with pyrimethamine alone, and with sulfadoxine alone were assessed over a 14-day period and were compared with those of each of the two parents and AS(PYR) (Table 1; Fig. 2). Each progeny clone was tested in this way twice. For almost all clones, the drug response phenotypes proved to be similar in each test. Exceptions occurred when, for example, parasites in one of the three drug-treated mice in a test underwent a course of infection different from that in the other two animals. In such instances, further tests were made until at least two successive tests gave comparable results in all mice. Drug responses were assessed over a 14-day period after drug treatment. The parasitemias on day 6 proved to be a clear measure of the different phenotypes, and for simplicity only these data are shown (Table 1).

With regard to S/P, three phenotypes were detected, a sensitive phenotype (similar to that of parent clone AJ), a highly resistant phenotype (similar to that of AS(50S/P)), and a nonparental low-resistance phenotype (like that seen in AS(PYR)). For pyrimethamine, two phenotypes were observed, sensitive (similar to AJ) or resistant (similar to AS(50S/P) and AS(PYR)). For sulfadoxine, three phenotypes were identified, a high-resistance phenotype (like that of AJ), a low-resistance phenotype (like that of AS(50S/P)), and a nonparental sensitive phenotype (like that of AS(PYR)). Four progeny (1107/14, 1109/11, 1384/10, and 1538/12) exhibited the sulfadoxine-sensitive phenotype, and all of these possessed a high resistance to S/P as well as resistance to pyrimethamine (Table 1).

Inheritance of chromosome-specific markers and QTL analysis. The inheritance patterns of the 31 markers and the drug responses of the 16 progeny clones are shown in Fig. 2. The marker distribution and genetic map data will be presented elsewhere.

The S/P and pyrimethamine responses of the progeny were compared. Figure 3B shows that progeny inheriting the AJ-type \textit{dhfr} and progeny inheriting the AS(50S/P)-type \textit{dhfr} segregate within distinct groups of pyrimethamine-sensitive and -resistant phenotypes. While all progeny that inherited the AJ-type \textit{dhfr} were S/P sensitive, progeny that inherited the AS

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{Assessment of the drug sensitivities of AS(SENS) \textbullet{}, AS(PYR) \textcircled{O}, and AS(50S/P) \textbullet{}. CBA/Ca mice were infected on day 0 and treated for 4 days with either 25/1.25 mg of S/P kg$^{-1}$ (A), 10 mg of pyrimethamine kg$^{-1}$ (B), or 150 mg of sulfadoxine kg$^{-1}$ (C). The data shown are the means ± standard errors from representative experiments.}
\end{figure}
Clones are listed vertically. Roman numerals refer to chromosome number, marker numbers are shown along the bottom, and markers are ordered such that the number of crossovers per chromosome was minimal (5). An asterisk marks the inheritance profile of P. chabaudi dhfr-ts (marker 6). Black boxes indicate inheritance of AS(50S/P)-type alleles, open boxes indicate inheritance of AJ-type alleles, and grey boxes indicate data not available. Drug phenotypes are shown on the right, where black boxes indicate the inheritance of a resistant phenotype, white boxes indicate the inheritance of a sensitive phenotype, and hatched boxes identify progeny with an intermediate phenotype.

(50S/P)-type dhfr are distributed across a range of S/P values, suggesting that at least two genes are required for S/P resistance. Therefore, the data were analyzed by QTL methodology to identify loci linked to the S/P resistance phenotype.

The results of the QTL analysis are shown in Table 2. For each marker, the difference between the mean log parasitemia of grouped progeny clones inheriting the AS(50S/P)-type allele was 0.73 on day 6 following treatment with 25/1.25 mg of S/P kg$^{-1}$, while the mean log parasitemia of all progeny inheriting the AJ-type allele was 0.82 (Table 2 and Fig. 3A). The difference between the two means (0.09) was insignificant ($P = 0.46$) at $n = 14$. It could be concluded, therefore, that marker 4 is unlikely to be linked to a locus conferring resistance to 25/1.25 mg of S/P kg$^{-1}$.

Following application of the Holm procedure to correct for false positives due to multiple testing, two loci showed evidence of strong linkage to drug responses. The AS(50S/P) allele of P. chabaudi dhfr (marker 6) on chromosome 7 was strongly linked not only to resistance to pyrimethamine (as expected) ($P < 0.001$) but also to resistance to S/P ($P < 0.01$) (Table 2 and Fig. 3B). In addition, mutant P. chabaudi dhfr was

![FIG. 2. Inheritance of 31 chromosomal markers among AJ, AS(50S/P), and 16 progeny clones from two AJ × AS(50S/P) crosses. Progeny clones are listed vertically. Roman numerals refer to chromosome number, marker numbers are shown along the bottom, and markers are ordered such that the number of crossovers per chromosome was minimal (5). An asterisk marks the inheritance profile of P. chabaudi dhfr-ts (marker 6). Black boxes indicate inheritance of AS(50S/P)-type alleles, open boxes indicate inheritance of AJ-type alleles, and grey boxes indicate data not available. Drug phenotypes are shown on the right, where black boxes indicate the inheritance of a resistant phenotype, white boxes indicate the inheritance of a sensitive phenotype, and hatched boxes identify progeny with an intermediate phenotype.](http://aac.asm.org/)

### TABLE 1. Drug resistance phenotypes of AJ, AS(PYR), AS(50S/P), and the AJ × AS(50S/P) progeny clones

<table>
<thead>
<tr>
<th>Clone</th>
<th>% Parasitemia (mean ± SE)$^a$</th>
<th>Phenotype$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated control</td>
<td>S/P (25/1.25 mg kg$^{-1}$)</td>
</tr>
<tr>
<td>AJ</td>
<td>40.2 ± 3.3 0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>AS(PYR)</td>
<td>29.6 ± 3.3 0.3 ± 0.2</td>
<td>3.8 ± 2.7</td>
</tr>
<tr>
<td>AS(50S/P)</td>
<td>31.4 ± 3.3 14.3 ± 3.4</td>
<td>15.0 ± 3.1</td>
</tr>
<tr>
<td>1107/10</td>
<td>44.0 ± 9.8 1.9 ± 1.9</td>
<td>10.3 ± 1.65</td>
</tr>
<tr>
<td>1107/14</td>
<td>27.8 ± 5.4 9.6 ± 3.2</td>
<td>18.4 ± 15.9</td>
</tr>
<tr>
<td>1108/8</td>
<td>38.3 ± 8.5 0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>1109/11</td>
<td>46.2 ± 3.7 23.0 ± 22.3</td>
<td>24.0 ± 21.4</td>
</tr>
<tr>
<td>1110/4</td>
<td>58.6 ± 1.3 40.9 ± 2.1</td>
<td>13.3 ± 4.6</td>
</tr>
<tr>
<td>1110/8</td>
<td>52.6 ± 0.5 0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>1110/10</td>
<td>36.7 ± 15.0 0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>1110/13</td>
<td>35.4 ± 11.6 0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>1351/2</td>
<td>18.5 ± 7.8 0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>1384/10</td>
<td>17.7 ± 9.3 10.0 ± 6.6</td>
<td>5.3 ± 0.5</td>
</tr>
<tr>
<td>1385/2</td>
<td>37.1 ± 11.1 0.2 ± 0.2</td>
<td>29.8 ± 23.6</td>
</tr>
<tr>
<td>1396/1</td>
<td>43.3 ± 9.0 19.9 ± 1.4</td>
<td>56.6 ± 3.2</td>
</tr>
<tr>
<td>1398/14</td>
<td>39.5 ± 7.6 14.6 ± 10.0</td>
<td>32.3 ± 1.7</td>
</tr>
<tr>
<td>1538/12</td>
<td>28.2 ± 2.4 17.7 ± 1.1</td>
<td>11.1 ± 6.7</td>
</tr>
<tr>
<td>1538/14</td>
<td>26.2 ± 7.0 24.5 ± 1.5</td>
<td>13.3 ± 2.1</td>
</tr>
<tr>
<td>1539/1</td>
<td>40.9 ± 7.4 39.2 ± 0.9</td>
<td>32.8 ± 1.0</td>
</tr>
</tbody>
</table>

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$^a$ On day 6 of the indicated treatment.

$^b$ Drug phenotypes were assessed over 14 days after drug treatment. R, resistant; S, sensitive; LR, low-level resistance.
linked strongly to sensitivity to sulfadoxine ($P < 0.001$). The differences between the mean log parasitemias of the two groups of progeny clones inheriting either the AJ or AS(50S/P) dhfr allele were highly significant in each analysis. In contrast, the AS(50S/P) allele of marker 19 (g6pd, chromosome 13) was linked to sensitivity to S/P (although not after Holm correction), to sensitivity to pyrimethamine ($P < 0.01$), and to resistance to sulfadoxine ($P < 0.01$). Marker 23 (pfcrk3) on this chromosome showed similar strong linkage to pyrimethamine sensitivity and sulfadoxine resistance before correction, although there was no association with S/P response.

Other loci which appeared to show association with drug responses before Holm correction were as follows. The AS(50S/P) allele of marker 88 (anonymous marker PfJ) on chromosome 4 showed linkage to S/P resistance. Conversely, the AS(50S/P) allele of marker 57 (Ag3035) on chromosome 5 was associated with sulfadoxine resistance but also with sensitivity to pyrimethamine and S/P. AJ-type alleles on chromosomes 5 and 13 showed a significant linkage with both S/P and pyrimethamine resistance and sulfadoxine sensitivity.

**DISCUSSION**

We have demonstrated here that stable resistance to S/P can be produced in the malaria parasite *P. chabaudi* by single-step treatment with this drug combination of a clone already resistant to pyrimethamine. The resistance was caused by a gene or genes distinct from *dhfr* or *dhps*, since neither of these genes contained novel mutations in the S/P-resistant mutant AS(50S/P) compared to the clone from which it was selected. Our results also demonstrate clearly that S/P resistance in this parasite does not necessarily depend on the parasite being resistant to pyrimethamine and sulfadoxine separately. This is shown by the appearance of S/P-resistant clones that were sensitive to sulfadoxine among the progeny of the cross between AS(50S/P) and AJ.

The genetic basis of resistance to S/P has not been investigated experimentally in *P. falciparum*, mainly because of the difficulties of testing the response to this drug combination in vitro. Sulfadoxine and pyrimethamine have a synergistic action against malaria parasites (10). However, it should be noted that sulfadoxine by itself has limited efficacy; when it was used alone for clinical treatment of *P. falciparum*, failure to clear parasites commonly occurred (26). Indeed, this is seen in our present work by the lack of sensitivity to sulfadoxine of the wild-type *P. chabaudi* clones AS(SENS) and AJ. Also, several previous studies using rodent malaria models have demonstrated that mutants resistant to pyrimethamine exhibit an increased sensitivity to sulfa drugs, due to an increased dependence on PABA (14, 26). This was evident in our present work in the increased sensitivity of pyrimethamine-resistant AS(PYR) to sulfadoxine in comparison to sensitive wild-type clones.

AS(50S/P) was derived from AS(SENS) in two selection steps, first with pyrimethamine to produce AS(PYR) (38), which in the present work was treated with S/P to produce AS(50S/P). The simplest explanation for the development of AS(50S/P)'s high S/P resistance is that successive mutations occurred at each step. The initial selection of AS(PYR) by pyrimethamine treatment resulted in a mutation in *P. chabaudi* *dhfr* in which Ser was replaced by Asn at position 106 (9), causing resistance to pyrimethamine. In the present study, we have shown that AS(PYR) also possessed a slight resistance to S/P and a marked decrease in resistance to sulfadoxine compared to AS(SENS). The selection of AS(50S/P) from AS(PYR) produced high S/P resistance and an increase in sulfadoxine resistance, although not to the level seen in wild-type parasites.

The precise mutation event or events causing the S/P resistance of AS(50S/P) are unknown. We have shown that there are no extra mutations in either *dhfr* or *dhps* in this mutant. However, our genetic analysis has shown that its resistance is probably determined by several genes. This can be deduced from the finding that progeny clones exhibiting various parental and nonparental combinations of resistance to S/P, sulfadoxine, and pyrimethamine were obtained in the cross. Certain patterns of resistance were apparent. Sensitivity to S/P was invariably associated with sensitivity to pyrimethamine and re-
TABLE 2. Analysis for linkage between inheritance of markers and drug resistance

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Marker</th>
<th>Mean difference in log parasitaemia with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>S/P</td>
</tr>
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<td>-0.08</td>
</tr>
<tr>
<td>2</td>
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<td>0.43</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
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</tr>
<tr>
<td></td>
<td>47</td>
<td>0.48</td>
</tr>
<tr>
<td>4</td>
<td>88</td>
<td>0.86** (NS)</td>
</tr>
<tr>
<td></td>
<td>89</td>
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<tr>
<td>5</td>
<td>57</td>
<td>-0.91* (NS)</td>
</tr>
<tr>
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<td>-0.83* (NS)</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
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<td>0.52</td>
</tr>
<tr>
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<tr>
<td></td>
<td>24</td>
<td>0.18</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>1.13*** (***)</td>
</tr>
<tr>
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<td>94</td>
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<tr>
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<td>4</td>
<td>-0.09</td>
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<tr>
<td>12</td>
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<td>0.25</td>
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<tr>
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<td>0.56</td>
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<tr>
<td></td>
<td>98</td>
<td>0.18</td>
</tr>
</tbody>
</table>

* Mean difference in log parasitaemia between progeny clones inheriting the AS(50S/P) versus the AJ allele of each marker. The difference between the means provides an estimate of the phenotypic effect of inheriting either allele. Positive values indicate that the AS(50S/P) marker allele is segregating with resistance, while negative values indicate that the AJ allele is segregating with resistance. The statistical significance of the difference between the two means was determined by (i) Student’s t test alone and (ii) Student’s t test followed by Holm-Bonferroni correction (shown in parentheses) (13). The analysis could not be carried out for marker 52 because no progeny inherited the AS(50S/P) allele of this locus. *, P < 0.05; **, P < 0.01; ***, P < 0.001; NS, not significant.

A major problem with genome mapping in P. chabaudi is the present lack of chromosome-specific markers. Our ability to detect loci other than those closely linked to a genetic marker was not optimal (5). Due to the limited number of markers tested, false-positive associations could be expected. Our analysis of the inheritance of 31 markers for linkage to three drug-resistance phenotypes resulted in 93 marker contrast analyses. Procedures to correct the genome-wide thresholds to account for the analysis of multiple markers and drug resistance traits resulted in loss of power. Nonetheless, the application of the Holm procedure to our t test analysis confirmed the strong likelihood that genes on chromosomes 7 and 13 are determinants of resistance to the drugs studied here.

S/P-resistant parasites have been selected in other rodent malarial species, i.e., those caused by Plasmodium berghei and Plasmodium yoelii, by using a continuous low-dose method, but in these instances the resistant phenotypes were not stable once drug pressure was removed (19, 20, 25). The fact that in the present study stable resistance in AS(50S/P) was obtained after a single course of drug selection suggests that a mutation in a single gene, or at most a few genes, was the cause of the novel phenotype. Therefore, the selection of S/P resistance in P. chabaudi observed in the present study could reflect the evolution of S/P resistance in P. falciparum, which arose quickly in areas where pyrimethamine resistance and, most probably, mutations in P. falciparum dhfr were already widespread (26).

We have shown here that the S/P resistance of AS(50S/P) is not due to mutation events in P. chabaudi dhfr additional to confirmed that mutant P. chabaudi dhfr (chromosome 7) causes pyrimethamine resistance, as expected from the earlier studies (9, 11, 27, 45). The analysis has also shown that this gene, or one or more genes closely linked to it, is a major determinant of S/P resistance and sulfadoxine sensitivity. This finding is consistent with the results reported for P. falciparum by Wang et al. (40), in which an unidentified gene closely linked to P. falciparum dhfr on chromosome 4 was linked to folate utilization and sulfadoxine response in vitro. In addition, our analysis suggests that a second locus for S/P resistance could be present on chromosome 4 and that loci for sulfadoxine resistance could be present on chromosomes 5 and 13. Further, despite the fact that the AJ parent was sensitive to both drugs, AJ alleles of loci on chromosomes 5 and 13 were associated with both S/P and pyrimethamine resistance among the cross progeny. The corresponding AS(50S/P) alleles were linked to sulfadoxine resistance, although AS(50S/P) has a low level of resistance to this drug. AJ has a slightly higher growth rate than AS(50S/P), and it is possible that genes on chromosomes 5 and 13 conferring growth rate advantages inherited from the AJ parent may have contributed to the level of resistance observed. It is also noteworthy that P. chabaudi dhps-pppk is located on chromosome 13 (6). While we have ruled out mutation in P. chabaudi dhps-pppk as a cause of S/P and sulfadoxine resistance, it is quite possible that a linked gene is involved. Also, while we have detected no evidence of P. chabaudi dhps-pppk or dhfr-ts gene duplication in this work by pulsed-field electrophoresis of chromosomes of sensitive and mutant parasites (data not shown), it would be of interest to determine whether there are differences in the levels of expression of P. chabaudi dhps in resistant and sensitive parasites which could be due to upregulation of expression of this gene.
Asn-106 or in P. chabaudi dhps. This does not, of course, rule out the possibility that mutations in these genes may be involved in other S/P-resistant mutants of P. chabaudi. While multiple mutations in both P. falciparum dhfr and P. falciparum dhps appear to confer S/P resistance in many P. falciparum isolates (15, 22, 23, 28, 29, 39), there are certainly exceptions which cannot be attributed to, for example, acquired immunity eliminating resistant forms. As a general rule, the resistance of a microorganism to a drug can be expected to have a variety of genetic causes, especially when resistance has arisen multiple times. Alternative mechanisms of S/P resistance, independent of mutations in P. falciparum dhps, may have been overlooked in P. falciparum due to the difficulties associated with studying the S/P response of this species in vitro. The ease with which S/P-resistant mutants can be selected in P. chabaudi, as well as the similarities in the basic biology of this species to that of P. falciparum, make it a very good model for studying the genetics of this as well as other types of malaria drug resistance (7).

ACKNOWLEDGMENTS

We thank Mike Barrett and Chris Janssen for the gift of the initial HindIII P. chabaudi Vectorette II library, Richard Fawcett and Ronnie Mouney for technical assistance, and Margaret Mackinnon for helpful advice.

This work was supported by the Medical Research Council of the United Kingdom.

REFERENCES

24. Pas另外 text content provided by Google Books, but the specific page number is not visible in the text. The text snippet is separated from the rest of the document. The text continues from here.
### ERRATUM

**Sulfadoxine-Pyrimethamine Resistance in the Rodent Malaria Parasite**

*Plasmodium chabaudi*

Karen Hayton, Lisa C. Ranford-Cartwright, and David Walliker

Institute of Cell, Animal and Population Biology, University of Edinburgh, Edinburgh EH9 3JT, and Division of Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ United Kingdom

Volume 46, no. 8, p. 2482–2489, 2002. Page 2487, Table 2: The data for chromosomes 8 to 14 should appear as follows.

<table>
<thead>
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<th>Marker</th>
<th>Mean difference in log parasitemia with:</th>
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<td></td>
<td>S/P Pyrimethamine Sulfadoxine</td>
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*NS* indicates non-significant differences.