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RESEARCH BRIEF

Plasmodium falciparum: Three Amino Acid Changes in the Dihydrofolate Reductase of a Pyrimethamine-Resistant Mutant

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Pyrimethamine specifically binds to and inhibits dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase (EC 1.5.1.3) (Ferone, 1970). Pyrimethamine resistant (Pyr^R) malaria whose DHFR had altered properties have been reported (Ferone 1970; Sirawaraporn *et al.* 1984; McCutchan *et al.* 1984; Walter 1986; Banyal and Inselburg 1986; Inselburg *et al.* 1987). We previously isolated a Pyr^R mutant of *Plasmodium falciparum,* FCR3-D8 (Banyal and Inselburg 1986) that was resistant to a 200× higher pyrimethamine concentration than the wild type strain FCR3. We report here, based on nucleotide sequencing data, that the DHFR produced by FCR3-D8 contains three nucleotide changes that caused three amino acid changes compared to FCR3.

FCR3-D8 was isolated by first growing parasites in the presence of progressively higher levels of drug followed by mutagenizing the parasite population with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and then selecting and finally cloning a parasite that was resistant to a still higher drug concentration. As parasites selected for growth at progressively higher drug concentrations were not cloned at each step in the selection there is, unfortunately, no assured lineage between the final cloned Pyr^R mutant and previously selected and cloned mutants (Banyal and Inselburg 1986). FCR3-D8 had no detectable levels of DHFR activity in extracts and the enzyme exhibited a reduced binding of methotrexate (5- to 10-fold) compared to the wild type. FCR3-D8 was, however, also shown to produce normal amounts of the normal size bifunctional DHFR-thymidylate synthase (TS; 5,10-methylenetetrahydrofolate: dUMP *C*-methyltransferase EC 2.1.1.45) parasite protein that contained normal TS activity (Banyal and Inselburg 1986; Inselburg *et al.* 1987). It has maintained its pyrimethamine resistance while being grown in culture without pyrimethamine for more than a year.

We recently reported the cloning, complete nucleotide sequence, and corresponding amino acid sequence of the pyrimethamine sensitive FCR3 DHFR-TS gene (Bzik *et al.* 1987). We have now cloned and sequenced an *Eco*RI genomic DNA fragment of FCR3-D8 that encoded the complete DHFR sequence and part of the TS sequence (Bzik *et al.* 1987). An FCR3-D8 *Eco*RI genomic DNA library was prepared in λgt11, and clones with the appropriate *Eco*RI fragment were detected by *in situ* hybridization with a probe prepared from the previously cloned sequence DHFR-E1 (Bzik *et al.* 1987). Those *Eco*RI fragments, as others previously isolated in the same way, extended from the *Eco*RI site in the center of the DHFR-TS gene into the 5′ flanking region. Those fragments invariably contained a deletion in the flanking region that was probably due to a recombinational event in the AT-rich flanking sequence (Bzik *et al.* 1987). A 1.9-kb *Eco*RI fragment from one such clone was subcloned into pUC18 (Yanisch-Perron *et al.* 1985). That fragment was then prepared for sequencing by being purified, self-ligated, and sonicated and the randomly generated fragments size-fractionated (Deininger 1983). The ends of 0.3- to 0.7-kb fragments were enzymatically repaired (blunted) and cloned into *Sma*I digested, alkaline phosphatase-treated M13mp8 (Messing and Vieira 1982). The M13mp8 clones were picked and every nucleotide on both strands in the DHFR encoding region and the junctional region of the bifunctional protein connecting the DHFR and TS domains was sequenced at least three times. The nucleotides sequenced were those between nucleotides 16 and 1307 which included 32 nucleotides in 5′ noncoding DNA and extended to the nucleotides encoding amino acid 419, or 98 amino acids into the TS sequence (Bzik *et al.* 1987). The sequence analysis was limited to that part of the DHFR-TS gene since the

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FCR3-D8 mutant had been shown to have a normal level of the DHFR-TS protein, which was of grossly normal size, and which exhibited normal TS activity while manifesting reduced DHFR activity and methotrexate binding (Banyal and Inselburg 1986; Inselburg *et al.* 1987). The results showed that three nucleotide changes, G to A at nucleotide 208; A to C at nucleotide 371; and T to C at nucleotide 716 (Figs. 1a–1f) that caused three corresponding amino acid changes, Asp to Asn at amino acid 54; Asn to Thr at amino acid 108; and Phe to Ser at amino acid 223 (Table I), had occurred in the region encoding the mutant DHFR.

We noted that the amino acid change of Asp to Asn at amino acid 54 of *P. falciparum* DHFR (Bzik *et al.* 1987) had been previously reported at the corresponding amino acid (amino acid 27) in the *Escherichia coli* DHFR (Villafranca *et al.* 1983). That change resulted in an *E. coli* DHFR with 1/300th the activity of the wild type. We had also seen a dramatic reduction in DHFR activity in FCR3-D8. Howell *et al.* (1986) noted that that mutant *E. coli* enzyme, but not the wild type enzyme, showed a depressed activity at pH 7.0 and a progressively increasing activity (approximately 10-fold) as the pH was decreased to 5.0. As we previously measured DHFR activity at pH 7.0 we therefore measured the pH effect on the malaria DHFR activity in parasite extracts as previously described (Banyal and Inselburg 1986) except we only measured the enzyme activity in freshly prepared rather than previously frozen extracts and we also used the 0.033 *M* succinic acid + 0.044 *M* imidazole + 0.044 *M* diethanolamine + 10 m*M* β-mercaptoethanol buffer used by Howell *et al.* (1986). We found that DHFR activity at pH 7.0 in wild type FCR3 or FCR3-D7, another Pyr^R mutant of FCR3 with an altered DHFR activity, was about 30% higher than, but proportionally the same as, the activity previously measured. We also now did detect some DHFR activity (5 to 10% of the FCR3 or FCR3-D7) in FCR3-D8. We did not find an increased DHFR activity at pHs 6.5, 6.0, 5.5, and 5.0 in any of the extracts. We interpreted our failure to find a pH-dependent effect caused by the Asp to Asn change in the FCR3-D8 DHFR to either the overall differences in the malaria and *E. coli* DHFR structures or to the two additional amino acid changes in the DHFR that may have suppressed the possible pH effect of this mutation. A second amino acid change (Asn to Thr) at amino acid 108 involved changing the wild type amino acid so it corresponded to the consensus amino acid at that position (Bzik *et al.* 1987). The third amino acid change from Phe to Ser at amino acid 223 was a unique change.

We previously noted that among Pyr^R mutants selected in several steps or in a single step those selected in a single step, after mutagenesis, generally showed the lowest levels of Pyr^R (Banyal and Inselburg 1986). As the drug concentration at which the mutants were selected, particularly at lower concentrations, should

TABLE I
The Locations of Amino Acid and Nucleotide Changes in the Mutant DHFR of the *Plasmodium falciparum* Pyr^R Mutant, FCR3-D8

DHFR amino acid number[a]	54	108	223
FCR3 amino acid	Asp	Asn	Phe
FCR3 nucleotides	GAT	AAC	TTT
FCR3-D8 nucleotides	AAT	ACC	TCT
FCR3-D8 amino acid	Asn	Thr	Ser

[a] The amino acid numbers, the corresponding nucleotides, and the comparisons with those elements in the DHFR from other sources is found in Figs. 2 and 3 in Bzik *et al.* (1987).

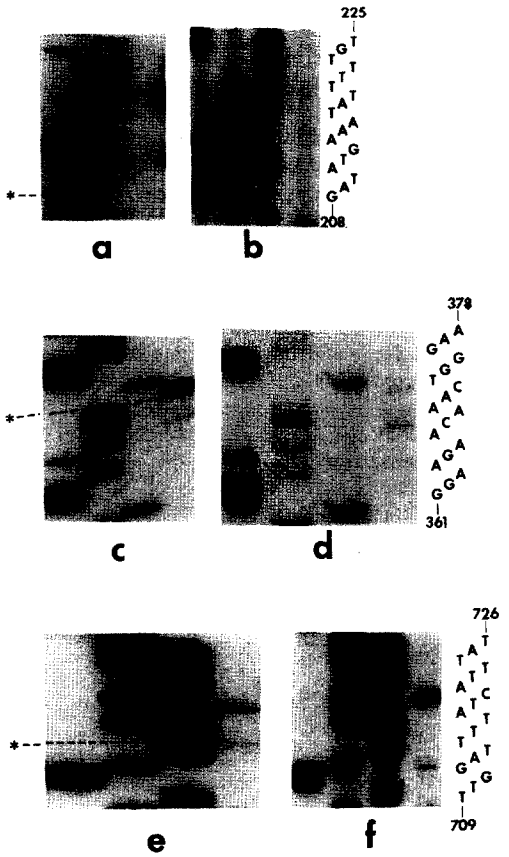

FIG. 1. A comparison of the nucleotide sequences in FCR3-D8 and FCR3 in those regions where mutations were observed. (a, c, e) Nucleotide sequences in FCR3-D8; (b, d, f) corresponding sequences in FCR3. The order of nucleotides in the autoradiograms is G, A, T, C. * marks the mutated nucleotide. Nucleotide numbering is based on the previously published FCR3 DHFR-TS DNA sequence (Bzik *et al.*, 1987). See Table I for the amino acid changes caused by these mutations.

not necessarily determine the maximum concentration the parasite will be resistant to, we found those results suggested the resistance to higher drug concentrations was probably most effectively achieved through multiple changes that led to the incremental increase in resistance. Depending on the mechanisms of resistance the multiple changes need not be localized in a single gene. The stability of the high levels of resistance after prolonged growth of the parasite in the absence of drug that we have observed could also be explained in terms multiple mutations conferring the observed resistance.

The mutations in the DHFR encoding region confirmed that mutations involving DHFR can be the cause of Pyr^R in malaria, an observation previously suggested by the findings of the altered DHFR properties in other Pyr^R parasites. A comparison of the mutations in naturally occurring Pyr^R mutants with those mutations in mutants selected by a single step mutation-selection process (Banyal and Inselburg 1986) should help in interpreting the role of multiple mutations in the DHFR gene in naturally resistant populations. The FCR3-D8 DHFR allele offers a potential selective marker for establishing a malaria transformation system. Such a system would assist in the dissection of the effects of the possible multiple mutational changes associated with drug resistance.

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