Auxotrophs of *Plasmodium falciparum* dependent on p-aminobenzoic acid for growth

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ABSTRACT The isolation of auxotrophic strains of a parasite offers new opportunities for studying parasitology. We have isolated cloned lines of *Plasmodium falciparum* that, unlike the parent line from which they were derived, rely on exogenous p-aminobenzoic acid (PABA) for growth. Isolation involved random mutagenesis of a cloned line of *P. falciparum* and subsequent selection of PABA-dependent parasites. Both parent and PABA-dependent clones were analyzed for PABA uptake and synthesis. Each clone takes up comparable amounts of PABA from the medium. The parent line, clone 3D7, can synthesize PABA de novo, whereas the PABA-dependent clones cannot. The requirement of exogenous PABA for growth by the auxotrophic strains coupled with their inability to synthesize PABA indicates that normal parasite growth can be completely supported by either synthesis or salvage. This work further clarifies the relationship between the availability of PABA and success of the parasite, an issue of debate from classic studies showing reduced parasite load in individuals on milk-fed diets.

The development of auxotrophic mutants with various supplemental requirements has provided essential tools for defining biochemical pathways in bacteria. As well as providing a general understanding of the biochemistry of the organism, these mutants have been used to define drug targets. Further, the generation of auxotrophic mutants of *Salmonella* has proven invaluable as a source of attenuated live vaccines (1, 2). In particular, *Salmonella* mutants defective in the aromatic pathway (i.e., synthesis of aromatic compounds including aromatic amino acids, ubiquinones, and folates) have proved the most effective attenuated vaccines compared with mutants defective in other metabolic pathways (3). The generation of auxotrophic mutants of malaria parasites potentially holds some of the same promise for parasitology that it holds for bacteriology.

We have elected to isolate auxotrophic mutants in the metabolic pathway for folate synthesis. A complete aromatic pathway (including synthesis of folates) exists in bacteria, fungi, and plants, but mammals have no corresponding pathway. *Plasmodium* has been found to contain at least the second part of the pathway. *Plasmodium* can synthesize folic acid from p-aminobenzoic acid (PABA), an intermediate in the pathway. The conversion of PABA to folate is a principal target for antimalarial drugs. These drugs include the potent antimalarial agents sulfonamides, which are chemical analogs of PABA. Also, relating to this pathway, pyrimethamine and cycloguanil are effective antimalarial drugs that inhibit the enzyme dihydrofolate reductase-thymidylate synthetase, essential for converting dihydrofolate to tetrahydrofolate (4).

The extent to which *Plasmodium* can synthesize PABA de novo is less clearly defined and remains controversial in malaria research. A requirement for exogenous PABA was indicated in early studies of cultivation of *Plasmodium* knowlesi (5). Milk diets, which are deficient in PABA, were found to correlate with the failure of several species of *Plasmodium* to grow in hosts (6–8). In these studies, PABA addition to the diet restored parasite growth. These observations combined with the effect of sulfonamides on parasites led to the conclusion that *Plasmodium* cannot supply their own needs for PABA through de novo synthesis. *Plasmodium* was thought to be unable to synthesize PABA, even though some of the tested parasite lines proliferated in hosts without supplemental PABA (9). This conclusion has been disputed in more recent studies. The majority of several tested *Plasmodium falciparum* lines grow at normal rates in cultivation without detectable levels of PABA or folates (10), indicating that the parasites are synthesizing the PABA they require. Also, Dieckmann and Jung (11) have detected four enzymes involved in PABA synthesis in extracts from *P. falciparum*-infected red blood cells. Despite this evidence even recent reports on the status of malaria research have not acknowledged the existence of a pathway for PABA synthesis (12). Here we show that a complete folate pathway exists in *P. falciparum* and that mutants can be produced with alterations in this pathway, such that their growth is regulated by addition of exogenous PABA.

MATERIALS AND METHODS

**In Vitro Culture of *P. falciparum***. *P. falciparum* strains were cultured in human red blood cells by the method of Trager and Jensen (13). Parasites were grown in a 3% CO2/2% O2/95% N2 gas phase. Before use in experiments, red blood cells were washed three times in PABA- and folate-free RPMI 1640 medium (GIBCO)/0.2% NaHCO3/20–40 mM Hepes/gentamycin at 40 mg/ml. Culture medium for the experiments consisted of the RPMI 1640 medium lacking PABA and folate with 0.2% NaHCO3, 20–40 mM Hepes, gentamycin at 40 mg/ml, and 10% (vol/vol) human serum (Interstate Blood Bank, Memphis, TN, pH 7.1). Normal serum contains trace amounts of PABA and folates (ref. 10; I.I. and S.R.M., unpublished observations). This PABA-deficient complete medium was used because it was thought that parasites selected under these conditions would possess a strict requirement for high levels of exogenous PABA.

**Mutagenesis of *P. falciparum***. Growing parasites, at a parasitemia (percentage of infected red cells) of 1.4% and hematocrit of 5%, were irradiated with 50, 200, 1000, and 5000 rads (1 rad = 0.01 Gy). Parasites were counted on Giemsa-stained thin smears 2 days after treatment, and the total number of parasites in the 5-ml cultures was calculated. Late-stage trophozoites and schizonts were lysed with sorbitol, according to Lambros and Vanderberg (14). The remaining ring-stage trophozoites were grown overnight and counted again by microscopy (day 3). The synchronization

Abbreviations: PABA, p-aminobenzoic acid; PRBC, parasitized red blood cell.

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protocol was repeated on day 3, and the remaining parasites were grown until day 5. Synchronized parasites were again counted by microscopy. Table 1 shows the number of parasites (and parasitemia) on the three dates in the cultures that had received doses of γ irradiation.

Isolation of PABA-Required P. falciparum. The 3D7 clone of P. falciparum was washed extensively with PABA-deficient RPMI 1640 medium and grown for seven cell cycles in PABA-deficient RPMI 1640 medium before selection for parasites requiring PABA. This strain grew at normal rates in the PABA- and folate-deficient medium. Parasitized red blood cells (PRBCs) (10⁶) were γ-irradiated with 50 rads. After irradiation, parasites were grown for one cycle under permissive conditions: PABA-deficient RPMI 1640 medium supplemented with PABA at 5 μg/ml. The selection procedure began by washing the irradiated parasites in PABA-deficient RPMI 1640 medium extensively, followed by synchronization with sorbitol (14). Greater than 95% of the PRBCs were ring-stage trophozoites. Parasites were suspended and grown in PABA-deficient RPMI 1640 medium for 21 hr. This amount of time is sufficient for progression of 3D7 ring-stage trophozoites to late-stage trophozoites; yet this amount of time is only half the time required to produce new ring-stage parasites. Control 3D7 cultures were grown in parallel to confirm that the parasites had reached late-stage trophozoites. Parasites that had matured to ring-stage parasites were washed repeatedly with RPMI 1640 medium. PRBCs were washed and grown in PABA-deficient RPMI 1640 medium until they reached 5–6% parasitemia. The selection procedure was repeated for five more cycles of synchronization, selection with sorbitol after growth in PABA-deficient RPMI 1640 medium, and expansion of surviving parasites. After the final selection, ring-stage parasites were additionally purified on a Ficoll density gradient and directly cloned by limiting dilution.

Growth rate of parental type and PABA-requiring clones in PABA-deficient RPMI 1640 medium plus or minus PABA at 5 μg/ml was tested in duplicate and assessed by microscopy. Extensively dialyzed human serum, processed as described by Milhous et al. (10), was used for these assays to increase the rate at which a difference in growth with and without supplemental PABA would be observed. The experiments were initiated with 1 ml of asynchronous parasites washed with PABA- and folate-free RPMI 1640 medium and suspended in PABA-deficient RPMI 1640 medium supplemented or not with PABA at 5 μg/ml. The cultures were maintained throughout the experiments at a 0.5–5% parasitemia and 5% hematocrit through appropriate dilutions. The number of parasites was determined by evaluation of Giemsa-stained smears and calculation of the total number. The growth rates were determined as described by Milhous et al. (10). Briefly, the growth rate equals the difference of the natural logarithms of the number of parasites observed at two time points divided by the duration of time (difference in time points). Relative growth rate experiments were repeated three times.

**Assay of PABA.** After cultivation in PABA-deficient RPMI 1640 medium supplemented with PABA at 5 μg/ml to a parasitemia of ~5% at 5% hematocrit, P. falciparum clones were washed and cultured for 48 hr in PABA-deficient RPMI 1640 medium to a parasitemia of 20–25%. The infected red blood cells were centrifuged at 500 × g and washed three times with Dulbecco’s phosphate-buffered saline. Packed cells (500 μl) were then removed and mixed with 20 μl of 2.5 mM p-hydroxybenzoic acid (as an internal standard) and 1 ml of distilled water. An aliquot was removed and assayed for protein by the method of Bradford (15). The suspension was placed in a boiling water bath for 10 min, mixed thoroughly for 1 min, and centrifuged in an Eppendorf microcentrifuge at 16,000 × g for 15 min. The pellet was discarded, and the boiling and centrifugation steps were repeated as above. The final supernatant was then lyophilized and stored at −20°C.

PABA content was measured by an adaptation of the method of Lindsay et al. (16). The entire lyophilized sample was dissolved in 50 μl of distilled water and injected into a HPLC. The HPLC conditions consisted of a 300 × 3.9 mm µ-Bondapack C18 column (5-µm particle size, Phenomenex, Belmont, CA), a mobile phase of 12% (vol/vol) acetonitrile/0.1 M citrate buffer, pH 3.5, flowing at a rate of 1 ml/min, and a detection wavelength of 266 nm. The PABA content could be normalized to 100% parasitemia because uninfected red blood cells incubated under identical conditions do not contain detectable amounts of PABA.

**Drug Inhibition Assay.** Clone 3D7 and the PABA-requiring clones, B5, D11, E6, and E10, were treated with sulfamethoxazole. These samples were processed according to a standard assay for sulfa drugs (10, 17). PRBCs with a parasitemia of 0.5% (mostly late-stage trophozoites) and a 4% hematocrit were washed repeatedly with PABA-deficient RPMI 1640 medium. Washed PRBCs were treated with different levels of sulfamethoxazole (in PABA-deficient RPMI 1640 medium with dialyzed serum) in 5-fold steps. The range of sulfamethoxazole was between 6.4 nM and 20 μM. The medium was replaced with fresh drug and medium at 48 hr and 1 μCi of [3H]hypoxanthine (20 Ci/ml; 1 Ci = 37 GBq; Moravek Biochemicals, La Brea, CA) per well was included to monitor growth. At 72 hr, samples were harvested on a Tomtec (Orange, CT) harvester model 96, and radioactivity was counted with an LKB Betaplate counter.

**RESULTS AND DISCUSSION**

Studies of auxotrophs of bacteria and yeast have shed light on several biochemical pathways, and their utility has opened the doors to molecular biology and attenuated vaccines. One example of the expanding use of auxotrophs is Salmonella mutants harboring deletions in the aromatic pathway. These mutants were initially used to define the biochemical pathway. Subsequently, these mutants were found to be ideal hosts for genetic transformation. Salmonella carrying de-

| Table 1. Growth of irradiated P. falciparum line 3D7 |
|-------------------|-------------------|-------------------|-------------------|-------------------|
| **Dose, rads** | **Parasit, %** | **PRBC, * no.** | **Parasit, %** | **PRBC, * no.** | **Parasit, %** | **PRBC, * no.** |
|-------------------|-------------------|-------------------|-------------------|-------------------|
| 0 | 1.4 | 3.5 | 4.3 | 3.5 | 4.3 | 21 |
| 50 | 1.4 | 3.5 | 3.9 | 20 | 3.7 | 18 |
| 200 | 1.4 | 3.5 | 4.2 | 21 | 2.8 | 14 |
| 1000 | 1.4 | 3.5 | 3.9 | 19 | 1.4 | 6.8 |
| 5000 | 1.4 | 3.5 | 1.8 | 8.8 | 0.4 | 1.8 |

Parasit, parasitized; Sync, synchronization.

*Number of parasitized red blood cells × 10^-6; hematocrit of cultures was maintained at 5%.

All late-stage parasites were lysed on these days as described.
fined deletions in the aromatic pathway are effective attenuated vaccines (1, 2) and carriers of heterologous antigens (18). Analogous mutants of protozoa may be equally useful. However, the approach to isolation of auxotrophs must suit the particular organism in question. The lack of definition of the PABA pathway and a stable transformation system in Plasmodium, including the reagents for transformation (i.e., genes in the PABA synthetic pathway), precludes direct genetic alteration as an approach. Here we show a more generalized approach is applicable to Plasmodium and provides a set of mutants with different changes in the pathway. We used a mutagenesis and negative-selection scheme analogous to that used by Inselburg and colleagues (19, 20) who isolated temperature-sensitive mutants of this parasite.

Gamma irradiation was used to induce mutagenesis, instead of nitrosoguanidine treatment (19) in the hope of preferentially producing deletions in the parasite DNA. Deletions are less likely to revert to wild type. We initially titrated the dose of γ irradiation and correlated it with the effect on growth rate (Table 1). Note that the parasites were synchronized to early ring-stage forms twice (see Materials and Methods) to enhance visualization of the effects of irradiation at early time points. Effects on the growth rate of P. falciparum were observed at ≥200 rads. We therefore treated parasites with 50 and 200 rads before selection to induce a limited number of mutations per parasite.

Our negative selection procedure, as in the Inselburg study (19), depends on the fact that sorbitol lysis kills actively maturing parasites without damaging early ring-stage forms. Hence, in a developmentally synchronized parasite population, only early-stage parasites that cannot mature under selective conditions survive. For auxotrophs requiring PABA, one synchronizes parasites in culture to the ring-stage form, allows growth in PABA-free medium for less than the time required for one cell cycle, and then lyzes actively developing parasites with sorbitol. This procedure effectively selects for parasites that mature slowly without PABA in the medium. One then must expand the number of parasites that grow normally with PABA addition by growing the parasites for several rounds of replication with nutrient addition. Several rounds of this procedure effectively enrich for parasites with a PABA requirement that grow normally in its presence. In our study parasites of the P. falciparum 3D7 line were selected for PABA requirement. Single parasites from the PABA-requiring enriched pool were cloned by limiting dilution. We purified a total of 18 clones from the two pools irradiated with different doses for further analysis.

The growth of several of the clones from the low-dose pool was tested with and without PABA to verify that PABA-requiring mutants had been selected. Several clones grew less well without PABA (or folates) as shown in Table 2. The morphology of the PABA-requiring clones appeared normal after cultivation in PABA-deficient medium, although the number of parasites was considerably less than those grown with PABA. There was a 3- to 19-fold stimulation of growth over 7 days of cultivation when PABA was added to the medium; this range suggests that the clones contain a variety of genotypes. Growth of the parental strain 3D7 is unaffected by the availability of PABA or folates in the medium, as observed for most P. falciparum lines (G.A.M. and T.F.M., data not shown; ref. 10).

The growth rates of the PABA-requiring clones vary over time in cultivation with deficient medium. The effect of the absence of PABA was most dramatic after day 5. The delay in the effect of PABA removal on growth rate is probably due to residual amounts of PABA in the cultures, as observed in other organisms. Growth rates were determined, as by Milhou et al. (10), for the period between day 5 and day 7 and are shown in Table 2. Comparison of the growth rates in medium supplemented with PABA between the parental strain and any of the clones (data not shown) shows no significant differences. Therefore, no slow-growing mutants were selected whose phenotype is independent of PABA.

This method is an efficient procedure for isolation of auxotrophs of P. falciparum. Two-thirds of the 12 screened clones required PABA for normal growth. This approach should be useful for isolation of auxotrophs in any pathway in Plasmodium in which an exogenous end product can be supplied.

The PABA-requiring clones B5, D11, E6, and E10 were screened for phenotypic and genotypic characters. Because chromosomal variations have been seen in other selected lines of the parasite, chromosome size and number of the PABA-requiring clones were compared with clone 3D7 by pulsed field electrophoresis. No clear variation was observed between strains (data not shown). The irradiation, therefore, did not induce major chromosomal rearrangements in the selected PABA-requiring clones. Further, the PABA-requiring lines show no variation from the parental line with regard to gametocyte production (data not shown).

PABA uptake and metabolism were analyzed in four PABA-requiring clones. No differences in PABA uptake, as measured by uptake of [3H]PABA from the medium, were observed between clone 3D7 and the four PABA-requiring clones (data not shown). Therefore, a defect in PABA uptake is not responsible for the auxotrophy. To assess de novo PABA synthesis, PABA content was assayed by HPLC in uninfected and P. falciparum-infected red blood cells, after incubation in PABA-deficient medium for 48 hr (Table 3). Although trace amounts of PABA are found in normal serum, PABA could not be detected (<1 pmol/mg) by this assay in uninfected red blood cells incubated in the PABA-deficient medium. Because PABA was not detectable in uninfected red blood cells, any PABA present in the cells must be synthesized de novo by the parasites. Red blood cells infected with clone 3D7 contained 19.88 ± 3.54 pmol/mg of protein. In contrast, PABA (<1 pmol/mg of protein) could not be detected in red blood cells infected with clone B5, D11, E6.

Table 2. Growth of PABA-requiring clones with and without PABA

<table>
<thead>
<tr>
<th>Clone</th>
<th>Parasites*</th>
<th>Growth rate†</th>
<th>Parasites*</th>
<th>Growth rate†</th>
<th>Stimulation,‡ fold</th>
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<tbody>
<tr>
<td>B5</td>
<td>10.8</td>
<td>0.07</td>
<td>58.8</td>
<td>0.63</td>
<td>5</td>
</tr>
<tr>
<td>D11</td>
<td>6.36</td>
<td>0.17</td>
<td>66</td>
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<td>E6</td>
<td>2.52</td>
<td>0</td>
<td>40.8</td>
<td>1.01</td>
<td>16</td>
</tr>
<tr>
<td>E10</td>
<td>5.76</td>
<td>0.38</td>
<td>55.2</td>
<td>1.06</td>
<td>10</td>
</tr>
<tr>
<td>D8</td>
<td>3.6</td>
<td>0</td>
<td>68.4</td>
<td>1.36</td>
<td>19</td>
</tr>
<tr>
<td>D9</td>
<td>4.8</td>
<td>0</td>
<td>70.8</td>
<td>0.86</td>
<td>15</td>
</tr>
<tr>
<td>B2</td>
<td>24</td>
<td>0.54</td>
<td>82.8</td>
<td>0.86</td>
<td>3</td>
</tr>
</tbody>
</table>

*Parasites equal total number of PRBC × 10^−7 in cultures maintained by dilution at 5% hematocrit and a range of 0.5–5% parasitemia on day 7.
†Average growth rate from day 5 to day 7, calculated as described.
‡Ratio of parasite number at day 7.
or E10. These data imply that, although the parental 3D7 strain can synthesize PABA, the four clones are deficient in the capacity to synthesize PABA. This inability to synthesize PABA is the most likely explanation for their auxotrophy.

The PABA-requiring clones appear to contain a mutation in the anabolic pathway for PABA production. We speculate that the mutations in our cloned parasite lines reside in the PABA synthetase gene. PABA synthetase is the final enzyme in synthesis of PABA and has been detected in extracts of P. falciparum cultures (11). It is the enzyme responsible for the conversion of chorismate to PABA. Chorismate is the common end product of the aromatic pathway. The PABA-requiring clones should not contain mutations in earlier steps in the pathway because chorismate is the common precursor to essential aromatic compounds without which the parasite would be unlikely to survive. In bacteria and fungi, these include aromatic amino acids, ubiquinones, and enterochelin. The possibility of mutations in the common aromatic pathway in these PABA-requiring clones, however, cannot be ruled out. Plasmodium may not rely on this pathway for synthesis of other aromatic compounds, especially in culture that is supplemented with high levels of aromatic amino acids at all steps in the selection.

The inhibition of plasmoidal growth by sulfonamides can be antagonized by PABA addition. It might, therefore, be expected that parasites that cannot synthesize PABA would be more sensitive to sulfonamides. The effect of a common sulfonamide, sulfamethoxazole, on the PABA-requiring clones was examined (Fig. 1). The PABA-requiring clones B5, D11, E6, and E10 and the parental 3D7 line were first grown in PABA- and folate-free RPMI 1640 medium plus dialyzed serum for 24 hr. After 24 hr there were no differences in appearance or growth between clone 3D7 and the PABA-requiring clones. The parasites were then treated with increased doses of sulfamethoxazole in PABA- and folate-free RPMI 1640 medium plus dialyzed serum for 72 hr. There are no differences between the IC<sub>50</sub> or the dose–response curves of wild-type 3D7 clone and any of the PABA-requiring clones. The sensitivities are similar between clone 3D7 and PABA-requiring clones, even though no PABA is detectable in the PABA-requiring clones after 48 hr of cultivation in PABA-deficient medium (see Table 3). This is a surprising result, given the mode of action of sulfa drugs and the observations with sulfonamide-resistant Plasmodium berghei (7). The ability to synthesize PABA, therefore, does not appear to affect susceptibility to sulfa drugs.

Our mutants respond differently to sulfonamides than a naturally occurring PABA-requiring line, Sierra Leone/CDC. Sierra Leone/CDC is sensitive to the sulfonamide sulfadiazine in medium with dialyzed sera analogous to our auxotrophs, yet this strain is resistant to high doses (IC<sub>50</sub>&gt;30 &mu;g/ml) in medium with normal sera (10). We have not observed this phenotype with our PABA-requiring clones (data not shown).

In summary, we have selected and cloned a set of P. falciparum lines that require supplemental PABA to grow. Biochemical analyses of the PABA-requiring clones show that although they take up PABA normally, they cannot synthesize PABA. The profile of sulfa drug sensitivity shows that these PABA-requiring clones have a different phenotype from the other P. falciparum line known to require PABA for growth. This study verifies the existence of a PABA synthetic pathway and should help clarify the effects of sulfa drug intervention on the parasite. The definition of this pathway may suggest further points for chemotherapeutic attack on folate metabolism. These cloned lines could be used to study development in the host and vector and to expand our understanding of folate requirements of the parasite. These lines could also be used to examine immune responses in the host, as PABA is the only known nutrient that can be used to regulate parasite growth through host diet.

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Table 3. Assay of PABA content in P. falciparum lines

<table>
<thead>
<tr>
<th>Strain</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
<th>Exp. 4</th>
<th>Average</th>
<th>SD</th>
<th>n</th>
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<tbody>
<tr>
<td>3D7</td>
<td>25.0</td>
<td>19.0</td>
<td>16.9</td>
<td>18.6</td>
<td>19.9</td>
<td>3.54</td>
<td>4</td>
</tr>
<tr>
<td>B5</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>3.54</td>
<td>3</td>
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<tr>
<td>D11</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
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<tr>
<td>E10</td>
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<td>&lt;1</td>
<td>&lt;1</td>
<td>3.54</td>
<td>3</td>
</tr>
</tbody>
</table>

Fig. 1. Concentration–response curves for sulfamethoxazole against the 3D7 clone (●) of P. falciparum and the derived PABA-requiring clones: B5 (○), D11 (●), E6 (△), and E10 (▲) with a 48-hr drug exposure before labeling with [3H]hypoxanthine for 24 hr.