Effect of Dietary \(p\)-Aminobenzoic Acid on Murine \textit{Plasmodium yoelii} Infection

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Plasmodia species, unlike humans, can utilize \(p\)-aminobenzoic acid (PABA) for the de novo generation of folate. Plasmodial enzymes for the synthesis of PABA via the shikimate pathway are being investigated as novel targets for malaria chemotherapy. We show that, despite the presence of biosynthetic machinery to synthesize PABA, \textit{Plasmodium yoelii}, a rodent malaria species, requires exogenous dietary PABA for survival. Mice fed low-PABA diets do not die from lethal doses of \textit{P. yoelii}. The initiation of a PABA-deficient diet after \textit{P. yoelii} infection is established leads to the clearance of parasites and subsequent resistance to infection by \textit{P. yoelii}. An intact immune system is not necessary for protection, given that mice with severe combined immunodeficiency were also protected by PABA-deficient diet. Our studies suggest that the PABA content in the diet will affect the host clearance of malaria parasites and may affect the efficacy of treatments that target the shikimate pathway.

Malaria affects >200 million people/year and is responsible for ~1–2 million deaths per year, most of which occur in children in sub-Saharan Africa [1]. Increasingly widespread drug resistance has curtailed the efficacy of inexpensive treatments, such as chloroquine and dihydrofolate reductase inhibitor–sulfapyridine (SP) [2]. Resistance to newer and more expensive antimalarials, such as mefloquine, has already been encountered, which underscores the need to identify low-cost antimalarials that will enhance prospects for general use in developing countries.

The results of epidemiological studies have shown that the incidence of lethal malaria is less than would be expected in children aged <1 year [3, 4]. Some researchers have ascribed this to the protective effects of maternal antibodies [5, 6], but other studies have suggested that diet or other factors might be important [7–10]. We have investigated the effect of dietary \(p\)-aminobenzoic acid (PABA) on the clearance of \textit{Plasmodium yoelii} infection by mice.

MATERIALS AND METHODS

\textit{P. yoelii} infection. A lethal strain of \textit{P. yoelii} (a gift from Ruth Nussenzweig, New York University School of Medicine, New York) was maintained by passage in BALB/c mice. \textit{P. yoelii} strains YM (lethal) and 17X (nonlethal) were obtained from Maria Mota (New York University School of Medicine, New York; strains originally obtained from David Walliker, University of Edinburgh, United Kingdom). For parasite passage, parasitemia levels were determined by staining fixed cells with 4',6'-diamidino-2-phenylindole hydrochloride and counting manually or by flow cytometry.

Male BALB/c, CD1, or Fox-Chase C.B.-17 SCID mice were fed normal rodent food (5001; Lab Diet). PABA-deficient food (AIN-76A; Harlen Teklad) was used for some groups. As required, this food was manually ground in a mortar and pestle and supplemented by the addition of PABA (150 \(\mu\)g/g), inositol (280 \(\mu\)g/g),...
powdered milk (200 g/kg), or purines in the form of DNA/RNA (10 g/kg). Mice were switched to specialized diets at predetermined times with respect to infection. All animal experimentation was conducted in agreement with guidelines set forth by Albert Einstein College of Medicine and Yeshiva University.

**Toxoplasma gondii and Trypanosoma cruzi infection.** BALB/c mice were injected intraperitoneally with 4 × 10⁶ organisms of cultured *T. gondii* RH strain. For *T. cruzi*, female A/J Cr mice were each injected intraperitoneally with 1 × 10⁶ organisms.

**RESULTS**

We found that mice fed a defined diet lacking PABA recovered from a lethal dose of *P. yoelii* (2 × 10⁶), whereas mice fed a normal diet died within 14 days of challenge (figure 1, top). Parasites were apparent in the blood of mice fed a normal diet by 7 days after infection (4.1% ± 3.8% parasitemia), and, by day 9, mice had fulminating parasitemia (36% ± 17%) and died shortly thereafter. Parasites were not detectable by Giemsa smear in mice fed PABA-deficient food on day 7 or 9. The presence of parasites was confirmed by the transfer of blood on day 9 from a mouse fed a PABA-deficient diet to another mouse fed normal food. This mouse died from infection 12 days later. Mice that survived *P. yoelii* infection were observed for 1 month while receiving PABA-deficient food and then returned to normal food for another month without any signs of disease. They were rechallenged with another lethal dose of parasites (2 × 10⁶) 2 months after the original challenge. All mice survived. In a separate experiment, mice rechallenged 1 year after the initial infection also survived, which indicates persistent protection.

A comparison of the normal rodent food and the PABA-deficient food revealed other differences beyond PABA content, including the absence of inositol and purines. Because PABA is not metabolized by mammals, the PABA content of “normal” rodent food is not monitored and was not available. Both foods contained folate. A variety of supplements, including powdered milk, were mixed with the PABA-deficient diet, but only the supplementation of food with PABA restored susceptibility to lethal infection (table 1), with a duration of survival comparable to that of mice fed normal food during malaria challenge.

Our initial strain of *P. yoelii* was obtained decades ago. To rule out that the dietary effect we saw was unique to our strain, we obtained the well-characterized reference strains *P. yoelii yoelii* YM (a lethal strain) and *P. yoelii yoelii* 17X (a nonlethal reference strain whose genome was recently sequenced) [23]. Mice inoculated intraperitoneally with YM (3 × 10⁶ parasites) and administered PABA-free food beginning the day of infection survived, whereas the supplementation of the PABA-deficient food with 150 µg/g PABA resulted in the death of all mice by day 9 (data not shown). The nonlethal *P. yoelii* 17X strain (3 × 10⁶ parasites, administered intraperitoneally), which is cleared by immunocompetent mice, behaved normally in mice given PABA-deficient food supplemented with PABA, with peak parasitemias of ~70% seen on day 15 and clearance of parasites by day 23 (figure 2). Animals given PABA-free food had detectable parasitemias, but peak parasitemia levels were lower (0.8% ± 0.1% on day 7), and parasites were cleared earlier (by day 14 vs. 23).

A PABA-deficient diet begun after *P. yoelii* infection (2 × 10⁴ or 2 × 10⁵ parasites) also protected mice from death (figure 3, top, and table 1). Mice fed PABA-deficient food 5 days before, the day of, and 3 days after infection all survived lethal challenge. Animals fed PABA-deficient food for 2 or 4
Table 1. Survival of *Plasmodium yoelii*-infected mice given different dietary treatments.

<table>
<thead>
<tr>
<th>Group</th>
<th>PABA free</th>
<th>Purines</th>
<th>Milk</th>
<th>Inositol</th>
<th>PABA</th>
<th>Treatment</th>
<th>Time to death, d</th>
<th>Survived/died (%)</th>
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</thead>
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<td></td>
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<td></td>
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<tr>
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<td>+</td>
<td></td>
<td>0–40 h</td>
<td></td>
<td></td>
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<td>13</td>
<td>1/5 (20)</td>
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<tr>
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<td>+</td>
<td>0–4 days</td>
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<td></td>
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<td>None</td>
<td>19</td>
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<tr>
<td>4</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>None</td>
<td>9.5</td>
<td>0/5 (0)</td>
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<tr>
<td>5</td>
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<td></td>
<td>0–4 days</td>
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<td></td>
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<td>15</td>
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<tr>
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<td>5–15 days</td>
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<td></td>
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<td>+</td>
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<td>8</td>
<td>+ +</td>
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<td>0–26 days</td>
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<td>None</td>
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<td>11</td>
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<td>9</td>
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**NOTE.** Male BALB/c mice, 4–6 weeks old, were injected with a lethal dose (2 × 10⁶ parasites) of *P. yoelii*. Mice were fed different formulated diets for the indicated times (Treatment). All mice were given a normal mouse diet for a minimum of 30 days after the termination of dietary treatment. No other deaths were observed. Inositol, 280 μg of inositol/g AIN-76A; milk, 200 g powdered milk/kg AIN-76A; NA, groups in which no mice died; PABA-free food, AIN-76A diet from Harlen-Taklad (see Materials and Methods); PABA, 150 μg of PABA/g AIN-76A food; purines, DNA/RNA (10 mg/kg).

* Mice were injected with a lethal dose of 2 × 10⁶ parasites.

days after parasite challenge (2 × 10⁶ parasites) were partially protected and had a prolonged time to death (table 1). Mice infected with a smaller lethal inoculum of parasites (2 × 10⁴) had an 80% long-term survival rate when they were fed the PABA-deficient diet for only 4 days. At 10 times the lethal dose (2 × 10⁵), 4 days of treatment was still therapeutic, and survival time was extended 2-fold. Mice could also be treated after parasites were visible in a blood smear. A 10-day course of PABA-deficient food given as late as 5 days after *P. yoelii* infection cured 100% of mice. The effects of a low-PABA diet did not appear to be specific to mouse strains, given that similar results were observed with BALB/c and CD1 mice.

Immunity to rechallenge was affected by the age of the animals, the dose of parasites used to challenge, and the level of parasitemia prior to dietary treatment. Three weeks after initial infection (2 × 10⁴ *P. yoelii* at age 4–6 weeks), animals were given normal food (figure 3, bottom). Ten days later, they were challenged with another lethal dose of *P. yoelii* that was 5 times the original challenge dose (1 × 10⁵). Mice that had received the PABA-deficient diet 5 days prior to or on the day of original infection were incompletely resistant (20% and 40% survival, respectively), but all mice originally treated 3 days after infection survived rechallenge. (The survival rate of mice was less than that seen in figure 1, top, because of their younger age at rechallenge [8–10 weeks vs. 4 months] and the larger dose used for rechallenge.)

The protective effects of a PABA-deficient diet do not require a functional immune system (figure 4). Young SCID mice (age 4–6 weeks) on the PABA-deficient diet remained asymptomatic after a lethal challenge of parasites. After replacing PABA-deficient food with normal food at day 21 after infection, the parasitemia recurred, which confirms the importance of an intact immune system in the complete clearance of parasites. Mice died within 3 weeks after the restoration of the normal

![Figure 2.](image_url) Mice infected with the nonlethal doses of *Plasmodium yoelii* strain 17X cleared parasitemia sooner with dietary treatment. BALB/c mice (4–6 weeks old; n = 5) were given p-aminobenzoic acid (PABA)–deficient food (squares) or PABA-deficient food supplemented with 150 μg/g PABA (diamonds) the same day as infection with 3 × 10⁶ parasites. Mean levels of parasitemia (percentage of infected erythrocytes ± SD) are shown.
Figure 3. Resistance to subsequent infections with *Plasmodium yoelii* is influenced by the timing of dietary treatment. BALB/c mice (4–6 weeks old) were given *p*-aminobenzoic acid (PABA)–deficient food 5 days before (boxes; day −5), the same day (circles; day 0), or 3 days after (diamonds; day +3) initial infection with $2 \times 10^8$ parasites (top). A control group was fed normal food (X). At day 30, surviving mice were switched from PABA-deficient food to normal food. Mice were rechallenged 10 days later (day 40 after initial infection; parasites; bottom). Mice were monitored daily. Mice with delayed dietary treatment were more resistant to reinfection.

Figure 4. *p*-aminobenzoic acid (PABA)–deficient diet protects SCID mice from *Plasmodium yoelii*. Fox-Chase C.B.-17 SCID mice (aged 4–6 weeks) were injected with a lethal dose of *P. yoelii* ($10^8$ parasites) and administered a PABA-deficient diet (squares) or a normal diet (X) for 21 days. Diet was begun on the day of injection. After 21 days, when all SCID mice receiving the normal diet had died, all SCID mice receiving PABA-deficient diet were placed on a normal diet. All mice developed recurrent malaria and subsequently died.

diet, and *P. yoelii* infection was confirmed by blood smears. The protective efficacy of a PABA-deficient diet in SCID mice is consistent with direct action on the parasite rather than on an enhancement of the immune response.

The requirement of exogenous PABA may be unique to malaria species, possibly because of the unique physiology of the erythrocyte. The infection rate of mice with lethal doses of *T. gondii*, *T. cruzi* (figure 5), and *Trypanosoma brucei* (data not shown) was not affected by the PABA-deficient diet. *Trypanosoma* species are known to utilize folate, and *T. gondii* lives within metabolically active cells, such as fibroblasts, which allows the organism free access to nutrients.

**DISCUSSION**

PABA is neither synthesized nor used by mammalian cells. The results of early in vivo studies showed that dietary PABA was important for a variety of species of malaria in rodent, human, and primate hosts [11–14], but these observations have not been pursued recently because of biochemical and genetic evidence that malaria species can synthesize PABA [15–17]. It has been well established that dietary PABA and folate reduce the efficacy of SP regimens in rodent models of malaria [18], as well as in cultured *Plasmodium falciparum* [19–21].

PABA is synthesized from phosphoenolpyruvate derived from glycolysis and erythrose-4-phosphate from the pentose phosphate pathway. The genes for enzymes of the shikimate pathway are present in the *P. falciparum* and *P. yoelii* genomes [22, 23] (http://www.plasmodb.org; see also a recent *P. yoelii* sporozoite EST project) [24]. Genes homologous to 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, chorismate synthase, shikimate kinase, and PABA synthetase can be identified, and these enzymes have been proposed as potential drug targets [15–17].

Although *Plasmodia* species can synthesize PABA de novo, our data and those of others suggest that parasites are unable to synthesize sufficient quantities to survive in vivo. This requirement of exogenous PABA appears to be unique to malaria species, perhaps because of the unique physiology of the erythrocyte. If malaria parasites are deprived of dietary PABA, they may eventually compensate by up-regulating de novo synthesis. In this case, one would predict that inhibitors of the shikimate pathway would be more effective. Because the PABA-free diet was so effective, we were unable to test whether glyphosate and diet were synergistic. Glyphosate was not an effective treatment for *P. yoelii*–infected mice fed normal food (data not shown).
A p-aminobenzoic acid (PABA)-free diet does not protect mice from infection with *Toxoplasma gondii* or *Trypanosoma cruzi*. BALB/c mice (aged 4–6 weeks) were infected with a lethal dose of *T. gondii* (top) or *T. cruzi* (bottom). In both cases, mice fed a PABA-free diet (squares) did not survive longer than mice fed a normal diet (X).

Figure 5. A p-aminobenzoic acid (PABA)-free diet does not protect mice from infection with *Toxoplasma gondii* or *Trypanosoma cruzi*. BALB/c mice (aged 4–6 weeks) were infected with a lethal dose of *T. gondii* (top) or *T. cruzi* (bottom). In both cases, mice fed a PABA-free diet (squares) did not survive longer than mice fed a normal diet (X).

It will also be interesting to examine the effectiveness of dietary treatment on strains of malaria that are sulfa resistant, particularly those in which the molecular basis of resistance is known. The infection rate of mice with lethal doses of *T. gondii*, *T. cruzi*, and *T. brucei* was not affected by a PABA-deficient diet. In *P. yoelii*, salvage appears to dominate relative to de novo synthesis and is likely to be more efficient for the parasite.

Folate salvage by malaria may be less efficient than PABA salvage [18] and does not efficiently rescue in vitro *P. falciparum* cultures from the effects of glyphosate, an inhibitor of EPSP synthase [15]. In our experiments, folate was present in the PABA-deficient food. The effects of dietary PABA intake correlate with reports that well-fed children frequently have more severe manifestations of malaria [9]. Similarly, breast-feeding is associated with protection from malaria as well as from a number of other infectious diseases, although the protective effects of breast milk against malaria are less evident in children with diets supplemented with other foods [9]. Breast milk is PABA-deficient; therefore, a strict breast-milk diet would not favor malaria.

In areas in which malaria is endemic, repeated exposure leads to protective, nonsterile immunity [25]. Epidemiological studies that have compared areas of high and low malaria transmission have suggested that the mortality rate is similar, despite differences in malaria exposure, which suggests that frequent exposure to malaria is partially protective and that measures that cannot completely eradicate malaria transmission may not significantly affect the overall morbidity and mortality rates [26].

A dietary intervention that attenuates malaria infection while allowing immune exposure may both prevent serious sequelae and facilitate the establishment of protective immunity [25]. Epidemiological studies correlating dietary PABA intake with malaria incidence in areas of endemcity, as well as studies of populations with diets with low PABA intake, are likely to establish whether a PABA-deficient diet affects the incidence of severe malaria. It may also be possible to develop strategies to degrade dietary PABA.

The restriction of the dietary intake of PABA may also enhance the efficacy of SP regimens, delaying the emergence of resistant parasites in areas where significant resistance has not yet precluded the routine use of SP for the treatment of clinical malaria. Sulfa drugs are PABA analogues that compete with PABA and inhibit dihydropterase synthase. In many areas of Africa, SP has become the first-line treatment for malaria, although the resistance rate is rising [2]. We predict that PABA-deficient food will enhance the efficacy of sulfa-containing regimens in patients similar to that seen for *P. falciparum* cultures [19–21]. The effects of dietary intervention on sulfa-resistant strains will be of particular interest. Furthermore, agents that prevent PABA salvage [27] may represent an alternate chemotherapy strategy that will enhance the efficacy of sulfa-containing regimens, as well as acting synergistically with novel agents designed to target the shikimate pathway.

Because both breast and powdered milk are inexpensive, nutritious, and low in PABA, there would appear to be no barrier to test whether dietary intervention, in addition to conventional chemotherapy, can prevent severe malaria symptoms or accelerate the rate of cure. Dietary influences on the malaria survival rate may be particularly relevant in areas of sub-Saharan Africa in which there is a significant prevalence of human immunodeficiency virus (HIV) infection among mothers. Although breast milk may be protective against malaria and other infectious diseases, breast-fed infants are twice as likely to acquire HIV from HIV-infected mothers [28]. Breast-feeding is not recommended for infants of HIV-infected mothers in the industrialized world but is common in Africa among HIV-infected mothers because of the positive nutritional and health benefits and because the cost of formula precludes routine use. Infant formula contains many nutritional supplements not
present in breast milk, and its widespread use could alter the epidemiology of severe malaria in children in areas where malaria is endemic.

Acknowledgments

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References

5. Fried M, Nosten F, Brockman A, Brabin BJ, Duffy PE. Maternal antibody and protection of African infants from malaria in-That's all for now. Is there anything else you'd like to know or discuss related to this topic?