In Vivo Selection for a Specific Genotype of Dihydropteroate Synthetase of Plasmodium falciparum by Pyrimethamine-Sulfadoxine but Not Chlorproguanil-Dapsone Treatment

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*Plasmodium falciparum* present in blood samples collected before and 3 weeks after treatment with either pyrimethamine-sulfadoxine or chlorproguanil-dapsone was analyzed for variants of the genes coding for the target enzymes of antifolate drugs, dihydrofolate reductase (DHFR) and dihydropteroate synthetase (DHPS). Fragments of the genes were amplified by polymerase chain reactions, and variants were identified by specific restriction endonuclease digestion. Treatment with either drug combination selected for the variants Ile51, Arg59, and Asn108 of DHFR, which have been associated with in vitro resistance to pyrimethamine and cycloguanil. The genotype Ser436, Gly437, and Glu438 of DHPS was selected by pyrimethamine-sulfadoxine but not chlorproguanil-dapsone treatment, showing that a combination of these three variants is important for in vivo resistance to sulfadoxine in the area studied.

The antifolate drug combination pyrimethamine-sulfadoxine has become the treatment of choice for uncomplicated falciparum malaria in parts of east and southern Africa, where chloroquine resistance is widespread. Recent data from a holoendemic area in northeast Tanzania suggest that resistance to this drug combination has developed there recently [1–3]. In 1988–1990, a single dose of pyrimethamine-sulfadoxine was used successfully in children in the area to clear parasitemia for up to 4 weeks, after which the impact of vector control on malaria incidence was assessed [4]. However, in January 1995, 50% of treated children showed recurrence of parasitemia 3 weeks after pyrimethamine-sulfadoxine treatment, and subsequently, an alternative antifolate combination, chlorproguanil-dapsone, was tested in a comparative trial with pyrimethamine-sulfadoxine [3]. There were significantly fewer parasitic children in the group treated with chlorproguanil-dapsone at 1, 2, and 3 weeks after treatment [3]. It had been hypothesized [5] that because of the rapid elimination of its two components (elimination half-times: chlorproguanil, 12.6 h; dapsone, 24.5 h [6]), chlorproguanil-dapsone was less likely than pyrimethamine-sulfadoxine (elimination half-times: pyrimethamine, 81 h; sulfadoxine, 16 h [7]) to select for resistance.

Blood samples were collected before and 3 weeks after treatment with either drug combination to study variant alleles of the antifolate target enzymes dihydropteroate synthetase (DHPHS) and dihydrofolate reductase (DHFR), which have been associated with in vitro resistance in standard lines of *Plasmo-
_Plasmodium falciparum_ [8–11] and samples from malaria patients [12, 13]. A preliminary survey of some of these blood samples showed that Asn at position 108 of DHFR, a variant that has been associated with in vitro resistance to pyrimethamine and cycloguanil (the active metabolite of proguanil), was selected in vivo by treatment with either drug combination [14]. In the present study, other known polymorphic residues of DHFR, that is, 16, 51, 59 and 164, and the polymorphic positions 436, 437, 540, 581, and 613 of DHPS were assessed to try to identify the molecular basis of the differential response to chlorproguanil-dapsone and pyrimethamine-sulfadoxine treatment. Nested polymerase chain reactions (PCRs) were used to amplify fragments of the gene coding for each enzyme, and variants were identified by specific restriction endonuclease digestion.

**Methods**

**Study population.** The comparative trial of chlorproguanil-dapsone and pyrimethamine-sulfadoxine was carried out in 1995 on 40–50 asymptomatic children <10 years of age in each of 12 villages near Muheza, Tanga Region, in northeast Tanzania [3]. The samples studied here were from children in 4 of these villages.

**Blood samples.** Samples were collected before and 3 weeks after treatment with either a single standard treatment dose of pyrimethamine, 1.25 mg/kg, and sulfadoxine, 25 mg/kg (Pharmamed, Malta), or chlorproguanil, 1.25 mg/kg (Imperial Chemical Industries, Macclesfield, UK), and dapsone, 2.5 mg/kg (ECHO International Health Services, Coulsdon, UK), on 3 successive days. They were collected by fingerprick directly onto filter paper–glass fiber disks as previously described [14]. The samples were sent by airmail to London, where they were processed for use in PCR [14].

**Polymerase chain reactions.** A 648-bp fragment of the _dhfr_ gene was amplified with primers M1 (5'-TTTATGATGGAACAAGTGTGC-3') and M5 (5'-AGTATATACATCGCTAACAGA-3'). The amplified product was further amplified in two separate reactions with either M3 (5'-TTTATGATGGAACAAGTGTGC-3') and F/ (5'- AAATTCTGTGATAAACCAACGGACC-TCTATA-3') (lower case bases are mismatches, see below), giving a 522-bp fragment containing all five polymorphic residues, or F (5'-GAAAAGTGAATTCCTCATGATGAAATTT-3') and M4 (5'-TTAATTCCCAAGTAAACTTAGAGCCATTCTGTC-3'), giving a 326-bp fragment containing residues 59 and 108.

A 711-bp fragment of the _dhps_ gene was amplified with primers R2 (5'-AACCTAAAACCGTCTGTCC-3') and R (5'-AATTGTTGATTTGTCCCAACA-3'). The amplified product was further amplified in each of three nest 2 reactions. Primers K (5'-TGCGATCTGTGTATAGATGAGGCTAC-3') and K/ (5'-CTAATACCGAGGTACATTTATGAAGAACAACGGACTATA-3') (lower case bases are mismatches, see below), giving a 438-bp fragment for identification of variants at positions 436 (Ala and Ser), 437, and 540. Primers J (5'-TGCGATCTGTGTATAGATGAGGCTAC-3') and K/ (5'-CTAATACCGAGGTACATTTATGAAGAACAACGGACTATA-3') (lower case bases are mismatches, see below), giving a 438-bp fragment for identification of variants at positions 436 (Ala and Ser), 437, and 540. Primers K (5'-TGCGATCTGTGTATAGATGAGGCTAC-3') and K/ (5'-CTAATACCGAGGTACATTTATGAAGAACAACGGACTATA-3') (lower case bases are mismatches, see below), giving a 438-bp fragment for identification of variants at positions 436 (Ala and Ser), 437, and 540. Primers K (5'-TGCGATCTGTGTATAGATGAGGCTAC-3') and K/ (5'-CTAATACCGAGGTACATTTATGAAGAACAACGGACTATA-3') (lower case bases are mismatches, see below), giving a 438-bp fragment for identification of variants at positions 436 (Ala and Ser), 437, and 540.

Nest 1 reactions contained NH₄ buffer, 2 mM MgCl₂, 200 μM each dNTP, 0.25 μM each primer, 1% Tween 20, and 1 U of Taq polymerase (Bioline, London) in a volume of 50 μL. The DNA template was a small piece of washed glass fiber membrane containing the blood sample [14]. The PCR program used was 1 cycle of 94°C for 3 min; 40 cycles of 94°C for 1 min, 50°C for 2 min, and 72°C for 2 min; and a final 72°C for 10 min. Nest 2 PCRs contained NH₄ buffer, 2 mM MgCl₂, 200 μM each dNTP, 0.25 μM each primer, 5% DMSO, and 1.6 U of Taq polymerase in 80 μL. One to two microliters of the nest 1 product was used as template for second nests. The PCR program for nest 2 reactions was 1 cycle of 94°C for 2 min; 5 cycles of 94°C for 1 min, 45°C for 2 min, and 72°C for 2 min; 35 cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min; and a final 72°C for 10 min.

**Restriction enzyme digestion.** Restriction sites were present that defined the DHFR variants Ala16 (NdeIII), Asn413 (Tsp509I), Ser70 (AlaI), Asn406 (BsrI), and Thr613 (BstNI). Where restriction sites were not present naturally, primers were designed with mismatches toward the 3' end to produce restriction sites. Primer F gave an XmnI site when Arg59 was present and primer F gave a DraI site with Leu164.

The enzymes used to identify DHPS variants were _MnlI_ (SacI/R), _MspI_ (Ala236), _Avir_ (Gly201), and _FokI_ (Glu540) in the K-K' fragment. Primer K gave an MwoI site when Ala236 was present. Primer J gave an HindIII site with Phe326 and an Hhal site with Ala313. The variants at 581 and 613 were not described by restriction sites. Primer L gave a BsrUI site when Ala581 was present and a BsiI site with Gly510. Primer L gave an MwoI site with Ala581 and a BsaWI site with Ser613 or Thr613, and an AgeI site with Thr613.

Restriction sites for some enzymes in nonpolymorphic regions of the amplified fragments acted as internal controls for digestion. Fragment M3-F/ contained an _NlaIII_ control site, six _Tsp509I_ control sites, and two _DraI_ controls. Fragment M4-F/ contained an _XmnI_ control site, and fragment K-K' contained an _MnlI_ control. Other control sites were created by mismatches in the primers: _AflI_ in M4, _FokI_ in K, _MwoI_ in K', and _BsrUI_ in L'.

Restriction enzyme digestion was done under the conditions specified by the suppliers (New England BioLabs, Hitchen, UK). The appropriate amplified fragments of DNA from standard strains of _P. falciparum_ were used as positive and negative controls in each batch of digestion. Products of digestion were separated on 1%–3% gels containing 2:1 agarose (UltraPure; Life Technologies, Paisley, UK) for agarose (Nuseive GTG; FMC Bioproducts, Rockland, ME).

**Sequencing.** A novel variant at position 436 of DHPS was identified by sequencing a fragment of a nest 2 PCR product, R2-K'. The fragment was sequenced in both directions by using R2 as the sense primer or an internal antisense primer _S' (5'-TAT-AGTTAATGTGATCATT-3')_, a thermosequenase dye terminator cycle-sequencing kit (Amersham Life Science, Little Chalfont, UK), and analysis on a DNA sequencer (ABI Prism 377; Perkin-Elmer, Foster City, CA).

**Statistics.** Prevalences before and after treatment were compared by χ² with Yates's correction or Fisher's exact two-tailed test where appropriate. P < .05 was taken as significant.

**Results**

Table 1 shows the variants of DHPS in parasites present in blood samples collected before treatment and 3 weeks after
Table 1. Variants of DHPS present in *P. falciparum* in samples collected before and after treatment with either pyrimethamine-sulfadoxine or chlorproguanil-dapsone.

<table>
<thead>
<tr>
<th>Time point</th>
<th>DHPS residue</th>
<th>436</th>
<th>437</th>
<th>540</th>
<th>581</th>
<th>613</th>
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<td><strong>Before treatment</strong></td>
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<td>Ser (20)</td>
<td>Ala Gly (11)</td>
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<td>Ser Ala (8)</td>
<td>Ala Gly (13)</td>
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<td>Ala (1)</td>
<td>Gly (6)</td>
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<td>Ala Cys (1)</td>
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<td><strong>3 weeks after pyrimethamine-sulfadoxine treatment</strong></td>
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<td>Ser (23)</td>
<td>Ala Gly (1)</td>
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<td>Ser Ala (8)</td>
<td>Ala Gly (22)</td>
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<td>Ser Cys (1)</td>
<td>Ala Gly (8)</td>
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<tr>
<td>Ser Ala (4)</td>
<td>Gly (12)</td>
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<td>Ala Cys (2)</td>
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<td><em>P &lt; .001</em></td>
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<td><strong>3 weeks after chlorproguanil-dapsone treatment</strong></td>
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<tr>
<td>Ser (19)</td>
<td>Ala Gly (7)</td>
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<tr>
<td>Ser Cys (1)</td>
<td>Ala Gly (8)</td>
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<tr>
<td>Ser Ala (4)</td>
<td>Gly (12)</td>
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<td>Ala Cys (2)</td>
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<td><em>P &lt; .001</em></td>
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NOTE. Ser, serine; Ala, alanine; Cys, cysteine; Gly, glycine; Lys, lysine; Glu, glutamate; 2 amino acids indicate samples with mixture of clones with different variants at that position. Underscores indicate variants associated with resistance to sulfadoxine in vitro. Prevalence of Ala436, Ala437, or Lys540 before and after treatment was compared with *Fisher’s exact 2-tailed test or *Yates’s corrected χ². Prevalence of Cys436 was too low for analysis.

Discussion

Previously, we have demonstrated selection by chlorproguanil-dapsone or pyrimethamine-sulfadoxine treatment of the Asn108 variant of DHFR [14], which has been associated with in vitro resistance to pyrimethamine and cycloguanil [8, 9, 12]. In the present survey, it was shown that in addition to selection for Asn108, the variants Ile51 and Arg59 of DHFR, which confer increased resistance to pyrimethamine in parasites with Asn108 [8, 9], were also selected by both drug combinations. There are no data on the molecular basis of resistance to chlorcycloguanil, the active metabolite of chlorproguanil, but data on the structurally closely related compound cycloguanil showed that strains with high resistance to both pyrimethamine and cycloguanil carried Asn108 and one or both of the Ile51 and Arg59 variants [8, 9].

Chlorproguanil-dapsone treatment did not select for any of the known variants of DHPS present in the local population of *P. falciparum*. In contrast, the DHPS genotype Ser436, Gly437, Glu540 was selected by pyrimethamine-sulfadoxine treatment. A possible explanation for this difference is that resistance to dapsone involves changes in other, untested, residues of DHPS because of the structural differences between sulfadoxine and dapsone. However, we favor the hypothesis that the difference in selection is related more to the pharmacokinetics of the drug combinations.

There is high malaria transmission in the area where the drug study was done, with a reinfection rate of 20% per week [4]. Superinfection occurs, with concomitant infections in the blood and liver. The parasites in the blood at 3 weeks may have been present at treatment either in the blood or in the liver, from which they emerged later, or may have resulted from new mosquito bites. Pyrimethamine-sulfadoxine, because
of the long lives of its two components, would be present over the 3 weeks in sufficient quantities to select for resistant alleles of DHPS and DHFR in the blood, liver, or both. In contrast, recent pharmacokinetic data showed that because of the rapid elimination of chlorproguanil from the body, effective levels of the chlorcycloguanil-dapsone combination (as measured against a pyrimethamine-resistant \textit{P. falciparum} line in vitro) were probably present in the plasma for no more than 6 days after treatment began [6]. Nevertheless, there was selection for DHFR variants on parasites in the blood or liver, or both, during this time. The chlorcycloguanil-dapsone combination cleared parasites in the blood at treatment (95% of children were aparasitemic at 1 week [3]). It is likely that the chlorcycloguanil-resistant parasites present in the liver at the time of treatment were not cleared and caused the parasitemia at 3 weeks. This lack of clearance could be because dapsone is ineffective against liver-stage parasites of human malaria (reviewed in [15]).

Acknowledgments

We are grateful to Chris Curtis, Jane Trigg, Hamidi Mbwana, and their team of village health workers who collected the blood samples as part of their work on the impact of vector control on malaria incidence.

References


Antigen-Specific Proliferation and Interferon-γ and Interleukin-5 Production Are Down-Regulated during Schistosoma haematobium Infection

Jane L. Grogan, Peter G. Kremsner, André M. Deelder, and Maria Yazdanbakhsh

Antigen-specific cellular immune responses were examined in persons previously infected with Schistosoma haematobium and who were, 2 years after chemotherapy, either free from infection (n = 17) or reinfected (n = 20). Proliferation to adult worm antigen (AWA) was significantly higher in uninfected than in reinfected subjects (P = .02), whereas responses to soluble egg antigen (SEA) remained low in both groups. Interleukin (IL)-5 production in uninfected persons in response to AWA and SEA was higher than in infected subjects (P = .05 and P < .001, respectively), while IL-4 and IL-13 release was similar in the 2 groups. Levels of interferon-γ to AWA and to SEA were inversely correlated with egg output (P = .03 and P = .02, respectively). These data indicate that the presence of schistosome infection leads to T cell proliferative hyporesponsiveness and that both typical Th1 and Th2 cytokines can be down-regulated by active infection.

In schistosomiasis, immunoepidemiologic studies have established a clear age-related resistance to (re)infection, with children being more susceptible than adults [1]. These studies have also indicated that antigen-specific IgE and eosinophilia are associated with resistance to reinfection [2, 3], implying that a bias toward Th2 might provide protection to invading parasites. Indeed, in persons with a resistant phenotype, Th0 clones with a high ratio of interleukin (IL)-4 to interferon (IFN)-γ seem to be predominant [4]. However, it has been shown that persons exposed to schistosomes who remain free from infection (the so-called endemic normals) produce high levels of IFN-γ in response to parasite antigens compared with infected subjects [5]. Recently, a major gene that determines the intensity of Schistosoma mansoni infection was localized on chromosome 5, containing genes encoding, among others, Th2 cytokines and a region regulating IgE levels [6].

Superimposed on the genetic predisposition to infection is the modulation of the immune response by parasites to prolong their survival within their human host. In Kenya, studies of S. mansoni–infected persons have indicated that parasite-specific IFN-γ and IL-5 production is higher after clearance of infection [7]. Moreover, in Schistosoma haematobium–infected persons, we demonstrated that a decrease in egg output was associated with an increase in proliferation and IL-4 production at 5 weeks after chemotherapy [8]. These data suggest that both Th1 and Th2 subsets are involved in the immune response to parasites and may be down-modulated during active infection.

Here we have examined the cellular immune responses in persons previously infected, to similar levels, with S. haematobium, who are now uninfected or reinfected 2 years after chemotherapy, to determine how immune responses are influenced by the long-term presence or absence of infection.

Materials and Methods

Study population. The study took place in an area in which S. haematobium is endemic in Lambarende, Gabon. A cohort of 110 persons infected with S. haematobium has been previously analyzed for their humoral and cellular immune responses before and 5 weeks after treatment with praziquantel (40 mg/kg) [8]. In the present follow-up study 2 years after chemotherapy, 57 persons (who could be traced and were willing to participate) were examined for presence of eggs in urine and were questioned on their water contact, as recently described [9]. From the 57, we excluded...