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PROGUANIL PLUS SULFAMETHOXAZOLE IS NOT CAUSALLY PROPHYLACTIC IN THE *MACACA MULATTA—PLASMODIUM CYNOMOLGI* MODEL

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Abstract. New drugs for causal prophylaxis of malaria are needed. A proguanil/sulfamethoxazole combination was investigated using a rhesus monkey model (*Macaca mulatta* infected with *Plasmodium cynomolgi*) to determine whether causal prophylaxis could be achieved. When a five-day regimen of proguanil (40 mg/kg/day) combined with sulfamethoxazole (100 mg/kg/day) was used, infection of all animals (6 of 6) was observed, with an extended prepatent period (median 40 days). Two control animals became infected on days 9 and 23 following sporozoite inoculation. Plasma concentrations indicated that proguanil and sulfamethoxazole were adequately absorbed and metabolized to cycloguanil and N_4-acetylsulfamethoxazole, respectively. Analysis of liver biopsy specimens demonstrated that the drugs were present two days following sporozoite inoculation but were not detectable one week later. Proguanil plus sulfamethoxazole does not eliminate exoerythrocytic-stage parasites in the rhesus monkey–*P. cynomolgi* model.

Antifolates have been popular chemoprophylactic agents against malaria because of the infrequency of side effects, low cost, and good compliance. Although resistance to single agents such as pyrimethamine and proguanil has made antifolates ineffective in many areas, proguanil combinations are often recommended for causal prophylaxis in areas of malaria drug resistance.[1,2] Proguanil plus sulfamethoxazole has recently been shown to provide effective prophylactic protection in human field trials, with marked synergy between the components.[3,4] The problem of relapsing in *Plasmodium vivax* infections following the end of doxycycline chemoprophylaxis (up to 30% infection within one month after ending doxycycline) was not seen with proguanil plus sulfamethoxazole.[5] It seemed possible that a proguanil combination might have increased causal prophylactic activity against relapsing forms of malaria.

The only established primate model of causal prophylaxis, *Macaca mulatta* infected with *P. cynomolgi*, was used to test this hypothesis. This simian parasite is biologically related to *P. vivax* in its relapse patterns and its response to standard antimalarial drugs.[6]

MATERIALS AND METHODS

Animal experiments

The methods of Schmidt and others were used for the *M. mulatta–P. cynomolgi* model.[6] Malaria-naive rhesus monkeys of either sex weighing between 2 and 5 kg were infected intravenously with $0.5–1 \times 10^6$ *P. cynomolgi* sporozoites on day 0. Proguanil (40 mg/kg/day) plus sulfamethoxazole (100 mg/kg/day) was given daily on days −2, −1, 0, +1, and +2. Daily blood smears were used to detect parasitemia until 100 days, at which time negative blood smears were assessed to indicate successful prophylaxis. Untreated control animals were used to insure the infectivity of the sporozoites and estimate the prepatent period.

Animals were housed as a closed colony in double-screened, open-air ventilated animal rooms. They were fed a standard monkey chow, supplemented three times a week with approximately 100 g of fresh fruit. Water was freely available via automatic watering devices. Animals were sedated intramuscularly with ketamine hydrochloride (5–10 mg/kg) during nasogastric administration of test drugs, liver biopsies, intravenous sporozoite injection, and blood sample collections. Daily blood smears were made without sedation from the margin of the ear using sterile, disposable, pediatric skin lancets. After the conclusion of each set of experiments, animals were cleared of any remaining parasites by the administration of chloroquine and primaquine. Curative treatment was

TABLE 1

Summary of parasitemia data for Macaca mulatta *monkeys infected with* Plasmodium cynomolgi *following multiple dosing with proguanil (PRO) plus sulfamethoxazole (SMX)*

Monkey	Drug*	Drug days†	Day blood smear was first positive
DA 312	40 PRO/100 SMX	−2–+2	40
DA 317	40 PRO/100 SMX	−2–+2	28
DA 319	40 PRO/100 SMX	−2–+2	40
DA 327	40 PRO/100 SMX	−2–+2	40
DA 330	40 PRO/100 SMX	−2–+2	44
DA 355	40 PRO/100 SMX	−2–+2	38
DA 320	None		9
DA 347	None		23

* Values are in mg/kg.
† Days are numbered from day 0 (sporozoite inoculation).

verified by consistently negative blood smears for 100 days after the last treatment. Routine preventive medicine and health assessment procedures for all nonhuman primates in the colony included semiannual physical examinations and tuberculin skin testing. This research was conducted according to the principles enunciated in the *Guide for the Care and Use of Laboratory Animals,* Institute of Laboratory Animal Research, National Research Council, NIH Pub. No. 85-23, and applicable Federal laws and regulations.

Drugs used included proguanil hydrochloride (Paludrine™; Imperial Chemical Industries, London, UK) and sulfamethoxazole (Ganitol™; Roche Laboratories, Nutley, NJ). Tablets of proguanil and sulfamethoxazole were crushed to a fine powder using a mortar and pestle. Drugs were individually weighed on an analytical scale and maintained in a dry sealed vial until just prior to administration. Drugs were suspended in approximately 25 ml of distilled water not more than 30 min prior to administration. Placement of a size 8 french disposable infant nasogastric feeding tube for drug administration was verified by aspiration of gastric contents through the tube or administration of 1–2 ml of distilled water through the catheter without subsequent coughing by the animal. Following administration of the drugs, the feeding tube was flushed with 15 ml of distilled water.

Blood samples for plasma drug determinations were obtained by venipuncture. After separation from red blood cells, the plasma was stored at −70°C until analysis. When testing proguanil plus sulfamethoxazole, open liver biopsies were done on one control and three monkeys that had received the drug combination on day 2 postinfection and the remaining control and three other monkeys that had received the drug combination on day 9 postinfection. Liver specimens were dissected to a wet weight of approximately 250 mg and stored at −70°C.

Drug analysis

Plasma and liver concentrations of proguanil and sulfamethoxazole and their principal metabolites, cycloguanil and N_4-acetylsulfamethoxazole, were measured by high-performance liquid chromatography. The drugs were analyzed on a Waters 840 chromatographic system (Millipore-Waters, Milford, MA) consisting of a Model 510 pump, Model U6K injector, and a Lambda-Max Model 418 spectrophotometer. Chromatographic separation of proguanil, cycloguanil, and the internal standard chlorcycloguanil were performed on a Spherisorb-5-Phenyl column (particle size 5 μm, 150 × 4.6 mm internal diameter; HPLC Technology, Cheshire, UK). The mobile phase consisted of methanol and water (29:71 v/v) containing 5 mM low ultraviolet pentane-sulfonic acid (Millipore-Waters). Absorbance was measured at 238 nm. The run time was less than 17.5 min. Plasma samples (0.5 ml) containing 200 ng of chlorcycloguanil were prepared for solid-phase extraction based on the method of Taylor and others.[7] The interassay and intraassay coefficients of variation for both proguanil and cycloguanil at 10 ng/ml were less than 10% (n = 6). Liver samples were homogenized with an equal volume of water and then ultrasonicated. Liver suspensions (100 μl) were precipitated with methanol (1 ml), centrifuged, and the supernatant (1 ml) was subjected to solid-phase extraction. The intraassay coefficients of variation for proguanil and cycloguanil at 200 ng/100 μl of liver suspension were 4.6% and 4.5%, respectively (n = 4).

Sulfamethoxazole, N_4-acetylsulfamethoxazole, and the internal standard sulfalene were separated on a Nova-Pak C18 column (particle size 4 μm, 150 × 3.9 mm internal diameter; Millipore-Waters). The mobile phase consisted of acetonitrile, water, and glacial acetic acid (18.5:81:0.5, v/v/v). Absorbance was measured at 268 nm. The total run time was less than 12 min.

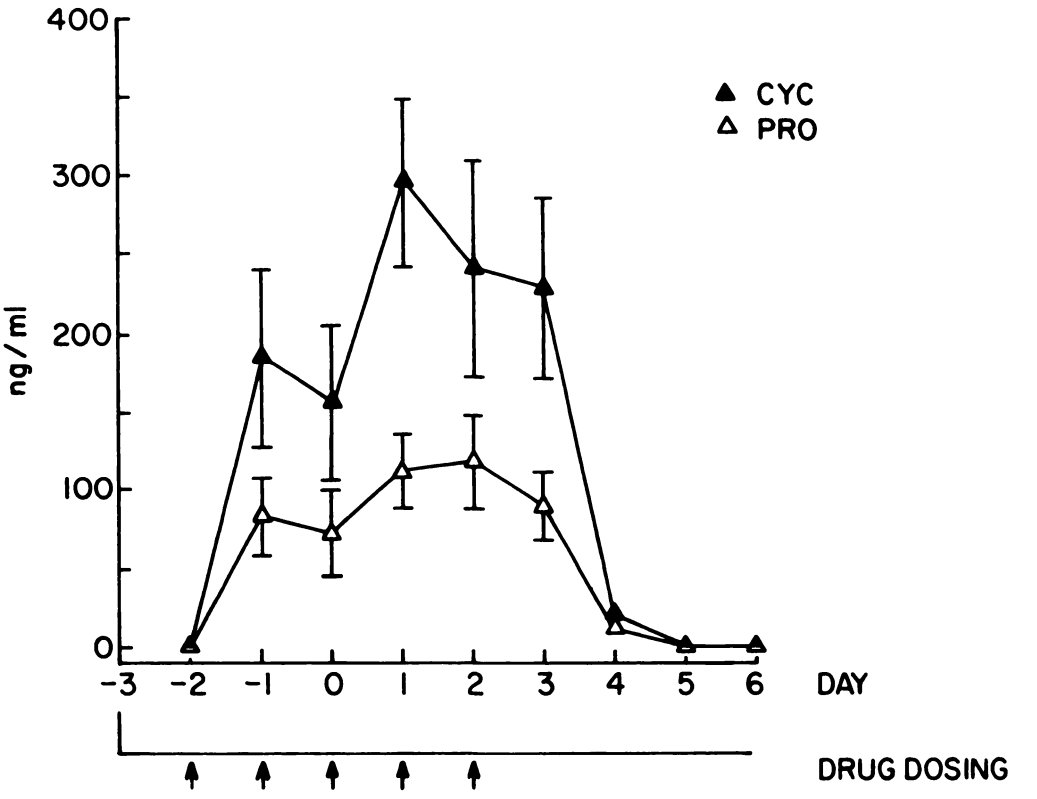

FIGURE 1. Minimum plasma concentration-time profiles (mean ± SEM) of proguanil (PRO) and cycloguanil (CYC) in rhesus monkeys (n = 6) infected with *Plasmodium cynomolgi*. **Arrows** indicate the days of drug administration.

Plasma samples (200 μl) containing sulfalene (5 μg) were extracted using liquid-liquid extraction at pH 5.6 based on the method of Edstein and others for sulfadoxine analysis.[8] The interassay and intraassay coefficients of variation for sulfamethoxazole (1 μg/200 μl) and N_4-acetylsulfamethoxazole (0.25 μg/200 μl) were less than 10% (n = 5). The detection limit was 20 ng/200 μl for both compounds. Liver samples were homogenized in an equal volume of water, ultrasonicated, and this suspension (200 μl) was extracted as described for plasma analysis. The intraassay coefficients of variation for sulfamethoxazole (0.5 μg) and N_4 acetylsulfamethoxazole (0.125 μg) were 2.8% and 4.8%, respectively (n = 4).

RESULTS

Monkeys receiving proguanil plus sulfamethoxazole for five days had their first detectable parasitemia from 28 to 44 days (median 40) following sporozoite inoculation (Table 1). The control monkeys who received no drug were first parasitemic on days 9 and 23. There was no obvious explanation for the unusually long prepatent period in one of the control monkeys. The usual prepatent period is eight days.[9]

The mean minimum plasma concentrations (drawn just prior to the next dose) of proguanil and cycloguanil are shown in Figure 1, which demonstrates little or no drug present after day 5. A similar result is seen in Figure 2, which shows the mean minimum plasma concentrations of sulfamethoxazole and N_4-acetylsulfamethoxazole. The liver concentrations (mean ± SD, n = 3) were 49.0 ± 53.3 μg/gm for proguanil, 15.3 ± 4.7 μg/gm for cycloguanil, 15.9 ± 8.6 μg/gm for sulfamethoxazole, and 24.1 ± 4.5 μg/gm for N_4-acetylsulfamethoxazole. No drug was detected in the livers of three drug-treated monkeys on day 9.

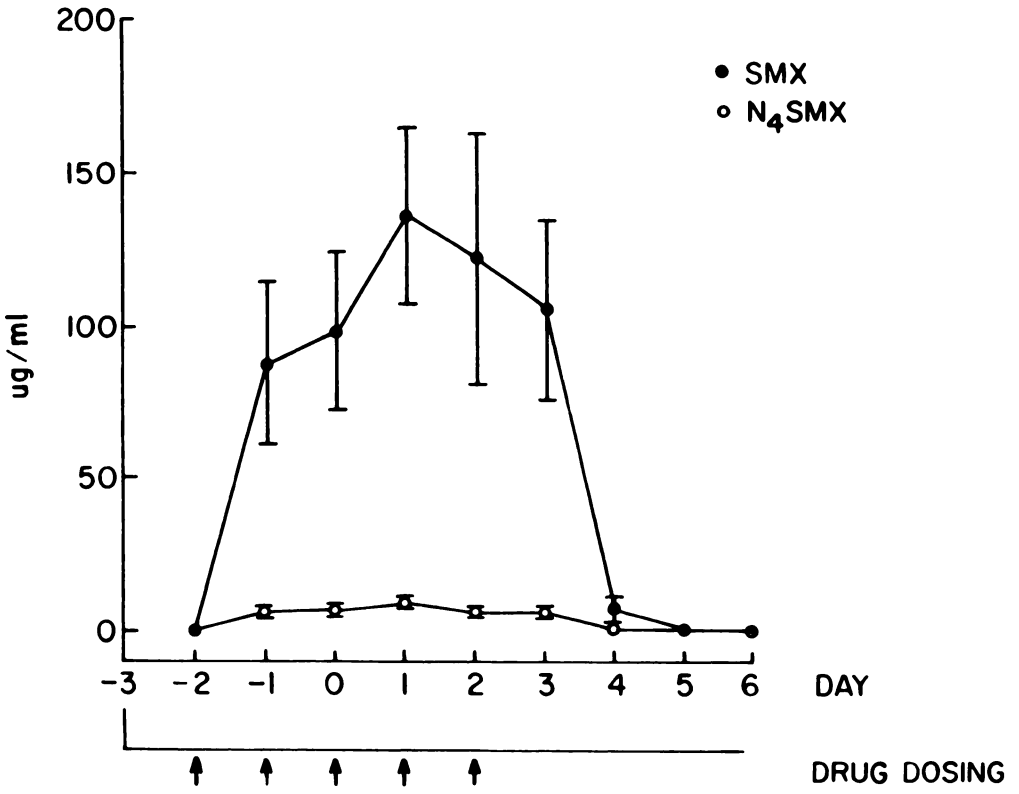

FIGURE 2. Minimum plasma concentration-time profiles (mean ± SEM) of sulfamethoxazole (SMX) and N_4-acetylsulfamethoxazole (N_4SMX) in rhesus monkeys (n = 6) infected with *Plasmodium cynomolgi*. **Arrows** indicate the days of drug administration.

DISCUSSION

With the *M. mulatta—P. cynomolgi* model used, we found no evidence of causal prophylactic activity using the combination of proguanil plus sulfamethoxazole. Previous work with 40 mg/kg of proguanil given for three days showed a delay in the onset of patency (mean 36 days) in three of three monkeys.[9] The delayed prepatent period without protection may be explained on the basis of two different populations of schizonts in the exoerythrocytic cycle as previously proposed by Bray.[10] The synergistic combination of proguanil plus sulfamethoxazole was inadequate to eliminate these tissue schizonts, which do appear to have some antifolate sensitivity.[11] One possible explanation might be that hypnozoites are not metabolically active enough to be killed by antifolates. Thus, the primary tissue schizonts that multiply rapidly after infection are killed, but the hypnozoites that undergo multiplication later escape the antifolate action.

The pharmacologic data presented can be approximately compared with previous studies in humans. Proguanil has been reported to be absorbed in humans and extensively metabolized to cycloguanil, the triazine metabolite that is responsible for the antimalarial activity.[12] Rhesus monkeys appear to convert more proguanil to cycloguanil than that found in humans with a cycloguanil:proguanil concentration ratio greater than 1.0, compared with less than 1.0 in the majority of humans.[13] The plasma elimination half-life of cycloguanil in rhesus monkeys (9.3 hr) is shorter than that reported for humans (15.1 hr).[14] The elimination half-life of sulfamethoxazole, however, is similar in the rhesus monkey and humans (9.2 hr versus 11.5 hr).[15] Both rhesus monkeys and man metabolize sulfamethoxazole to its principal N_4-acetylated metabolite. The five-day drug regimen used did not produce measurable concentrations of drug at a time when merozoites would be expected to be released from the liver.

The data presented here are in conflict with some suggestions of causal prophylaxis from human field trials.[3,4] This could be explained by the intrinsic differences in the human and monkey tests. *Plasmodium vivax* may have a different drug sensitivity pattern than *P. cynomolgi*. In field trials with humans, subjects are generally exposed to small intermittent sporozoite inoculations by mosquitoes during long periods of continuous drug administration. The *M. mulatta–P. cynomolgi* model uses a large artificial sporozoite inoculation during a brief drug course. Although the primate model system used here was successful in screening 8-aminoquinoline drugs similar to primaquine, it has limitations for the causal prophylactic assessment of antifolates.[6] Care must be taken when interpreting data from animal models of malaria infection to classes of drug for which they were not originally developed.

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