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In Aotus infected with drug resistant P. falciparum, chloroquine resistance (CQR) was reversed by chlorpromazine and prochlorperazine. Both water-insoluble and soluble forms of a bisquinoline were not curative. Three trioxanes were as effective as arteether. Azithromycin, but not doxycycline cured infections. Drug resistance was not induced to two pyrimethamine analogs. A metabolite of halofantrine was no more active than the parent drug. CQR of the AMRU-1 strain of P. vivax, was confirmed; a primaquine analog (1.0 mg/kg x 3) only cleared parasitemias but cured with chloroquine. Primaquine alone or with chloroquine had little activity. A DNA vaccine produced the highest antibody titers after 4 intradermal immunizations. No gamma interferon was produced in Aotus by Interleukin-12. Multiple rechallenges with the FVO strain yielded sterile immunity. MSP-1, a DNA erythrocytic vaccine did not protect against challenge. Sta. Lucia-falciparum blood & sporozoite stages induced infections. A DNA pre-erythrocytic have yet to be evaluated, 3D7 clone sporozoites were not infective.

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Richard N. Rosnan

June 12, 1996

PI's - Signature Date

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INTRODUCTION

The essence of the problem addressed in this 5-year final report is: 1) to evaluate the potential antimalarial activity of drugs in the pre-clinical model of Aotus lemurinus lemurinus (Panamanian night monkey) experimentally infected with Plasmodium falciparum or P. vivax, and 2) to use this model to test recombinant DNA malaria vaccines. Drug evaluation studies were supported by the U. S. Army, while the vaccine studies received support from the U. S. Navy Malaria program. Studies with this model were initiated in 1976 at Gorgas Memorial Laboratory, Panama. Due to the drug resistance exhibited by the highly pathogenic P. falciparum parasites in Asia, Africa, and Latin America, it is essential that new drugs be evaluated in the preclinical Aotus model for their potential usefulness against human infections.

Initially, antimalarial drug studies used the Colombian Aotus as the experimental host (1, 2). In the mid 1970's embargoes imposed by South American countries on the exportation of monkeys seriously restricted the use of Aotus for biomedical research in the United States. Panamanian Aotus were available at Gorgas Memorial Laboratory, Panama, and the project transferred here in 1976. Diverse avenues of research have been pursued in attempts to identify effective new antimalarial drugs. Three strains of P. falciparum, Vietnam Smith, Uganda Palo Alto, and Vietnam Oak Knoll, had been adapted to Panamanian Aotus. These strains exhibit diverse susceptibility and/or resistance to standard antimalarial agents. The course of untreated infections in Panamanian Aotus has been characterized and compared with that in Aotus of Colombia (3). Overall, the virulence of these strains was less in Panamanian than in Colombian owl monkeys, as indicated by lower mortality rates of Panamanian monkeys during the first 30 days of patency. Maximum parasitemias of the Vietnam Smith and Uganda Palo Alto strains were, however, significantly higher during the first 15 days of patency in Panamanian than in Colombian owl monkeys. These quantitative differences in infection parameters between Panamanian and Colombian owl monkeys have not invalidated the use of the former for the evaluation new antimalarial drugs.

Numerous candidate antimalarial drugs of diverse chemical classes have been evaluated against trophozoite-induced infections of one or more P. falciparum strains during the course of these contracts. In seeking alternatives to primaquine, two 8-aminoquinolines proved to be active against the blood stages of P. falciparum (4,5). Desferrioxamine, an iron-specific- chelating agent, was shown to suppress parasitemias of the

virulent Uganda Palo Alto strain of P. falciparum (6). The in vitro activity of two halogenated histidine analogs was not confirmed by evaluation against P. falciparum infections in owl monkeys (7).

Chloroquine-resistance of P. falciparum represents the greatest challenge in developing effective antimalarial drugs. Reversal of chloroquine-resistance in P. falciparum, in vitro, was achieved by the co-administration of verapamil (a calcium channel blocker) plus chloroquine (8). Other in vitro studies have shown that there is a significantly greater efflux of chloroquine from erythrocytes containing falciparum parasites resistant to chloroquine than from red cells parasitized by chloroquine-sensitive falciparum malaria (9). Calcium channel blockers appear to prevent this active efflux of chloroquine, thus allowing the drug to accumulate to parasiticidal levels.

Based upon the success of in vitro reversal of chloroquine-resistance, trials were initiated to determine if resistance could be reversed in Aotus infected with the chloroquine-resistant Vietnam Smith strain of P. falciparum. Six calcium channel blockers, or similarly acting drugs, were co-administered with chloroquine in diverse regimens. The desideratum of chloroquine-resistance reversal was administration of a single course of treatment, with parasite clearance and infection cure. Suppression of parasitemia was obtained during an initial course of treatment, but parasite clearance and cure occurred in some instances only after re-treatment. Such infection parameters were similar to those in monkeys with self-limited infections and cure could be attributed to acquired immunity.

Limited trials with desipramine, Norpramin , a tricyclic psychotropic drug, demonstrated the feasibility of reversing chloroquine-resistance in vivo (10). Parasite clearance was obtained, but the infection was not cured.

Subsequently, in vivo reversal of chloroquine resistance was obtained with combinations of chloroquine plus chlorpromazine or prochlorperazine. Such reversal was exhibited by rapid suppression and clearance of parasitemia, resulting in infection cure without retreatment (11).

Evaluation of two oil-soluble derivatives of artemisinin, artemether and arteether, demonstrates that both possess similar activity to cure infections of a multi-drug resistant P. falciparum strain in Aotus.

Both the purpose and methods of approach of the present work remains essentially unchanged since 1976, viz to ascertain the antimalarial activity of drugs against P. falciparum infections in Aotus. The method of approach may vary on an ad hoc basis, such as administering a combination of drugs.

The long term goal of the second part of this project is to develop fully protective DNA vaccines that induce protective immune responses against the sporozoite, liver and erythrocytic stages of P. falciparum. If successful, it will establish for the first time that DNA vaccines can protect non-human primates, a critical step forward using DNA vaccines in humans.

Vaccines are aimed at inducing immune responses that disrupt the complex cycle of the parasite at one or more points: anti-sporozoite antibodies that prevent invasion of hepatocytes; cytotoxic T lymphocytes, cytokines, and antibodies that eliminate infected hepatocytes; antimerozoite antibodies that prevent invasion of erythrocytes; antibodies that neutralize parasite exoantigens known to induce harmful cytokine responses; antibodies that attack infected erythrocytes; cytokines that kill parasites within erythrocytes; and, anti-sexual stage antibodies that prevent the development of sporozoites in the mosquito.

Previous trials of malaria blood stage vaccine have shown that the Panamanian Aotus P. falciparum model to be suitable for this purpose. (12, 13, 14)

BODY

I. Experimental Methods

The first intent of this project is to evaluate the potential antimalarial activity of drugs, or combination thereof, in the preclinical model of Aotus experimentally infected with P. falciparum (or P. vivax). Specifically, the vertebrate host is Aotus lemurinus lemurinus, the Panamanian night monkey. These animals are either feral, laboratory adapted or laboratory born. No naturally acquired, human plasmodium infection has been reported in Aotus. The Vietnam Smith/RE strain of P. falciparum was adapted to Aotus of Colombian origin in 1971 (1) and in Panamanian Aotus in 1976. (3). The course of untreated infections, essential for comparison with treated infections, has been documented in Panamanian Aotus (3).

This plasmodium strain is resistant to maximally tolerated doses of chloroquine, pyrimethamine, and quinine (2).

To initiate an experiment, infected blood (with 2.5% sodium citrate as the anticoagulant) from an untreated Aotus was diluted appropriately in chilled saline (0.85%), such that each milliliter contained 5,000,000 parasites. This amount was inoculated into the saphenous vein of experimental and control monkeys.

Blood films, prepared and examined daily beginning on the first post-inoculation day, were stained with Giemsa. Parasitemias were evaluated as follows: negative, if no parasites were detected on a thick blood film after examination for at least 5 minutes; <10 parasites per cmm, if positive only on the thick blood film; parasite enumeration was by the Earle-Perez method and reported as the number of parasites per cmm. (15)

Blood films from untreated Aotus, serving as passage and/or control subjects, were prepared and examined daily during the primary patent period, and daily thereafter for at least three consecutive days after parasites could last be detected on thick blood films. When parasitemia had cleared, films were made and examined twice weekly until a total of 100 negative days had been recorded. If recrudescence occurred, blood films were obtained again on a daily basis.

Parasitemias were evaluated daily during the treatment period and until blood films were negative for at least seven consecutive days. The frequency of smearing was then reduced to two times per week (Monday and Thursdays or Tuesdays and Fridays). If no recrudescences occurred during a 100 day examination period, the infection was considered to have been cured.

Drug doses were calculated as mg base per kg of body weight. Stock solutions of water soluble compounds, at appropriate concentrations, were prepared with distilled water and stored at 8° C for the treatment period. If a compound was water insoluble, a suspension of the requisite amount of drug was prepared daily with 0.3% methylcellulose (in distilled water).

Oral administration of drugs was by gastric intubation with a 14 French catheter. The total volume of fluid administered, drug solution or suspension, and rinse was 14 ml.

The second intent of this project is to ultimately evaluate recombinant vaccines against the blood and sporozoite stages of P. falciparum and against the blood stages of P. vivax in the Panamanian Aotus model. Prior to actual anti-parasitic experiments various routes of administration of a candidate vaccine must be tried so as to produce significant antibody levels. These trials will be detailed in the appropriate sections, as will other experiments associated with the Navy Malaria program.

II. Results

A. Drug Evaluation

1. In vivo reversal of chloroquine resistance

a. WR 035941AB (BN: BL18130), protriptylene
WR 149557AB (BN: BM06813), tetrandrine

This study was a continuation of trials to reverse chloroquine-resistance in vivo by the concomitant administration of the experimental drug plus chloroquine. Prior to testing in infected monkeys, the drug combinations were evaluated for toxicity in malaria-cured monkeys, as determined by body-weight changes. There was no evidence of drug intolerance, such as vomiting, anorexia, or loss of motor function. Within one month after the drugs had been administered, the monkey body weights were essentially equal to the pre-experimental levels. The drug combinations were considered to be non-toxic and experiments initiated.

When Vietnam Smith/RE developing infections were treated with WR 035941 (19.0 mg/kg) plus chloroquine (20.0 mg/kg) for seven days, one infection was cured and a recrudescence occurred in two monkeys. Retreatment with WR 035941 (38.0 mg/kg) plus chloroquine cured the infection in one of two Aotus.

Primary treatment with WR 149557 (15.0 mg/kg) plus chloroquine (20.0 mg/kg) for seven days only cleared parasitemias in 3 of 3 monkeys, while retreatment with WR 149557 (30.0 mg/kg) plus chloroquine cured the infection in 1 of 2 Aotus.

b. WR 002158AJ (BN: BL 50610), promethazine

Based upon anecdotal observations in Africa, the concomitant administration of promethazine (Phenergan), an antihistamine, plus chloroquine, at least ameliorates P. falciparum infections. In a previous trial using the Aotus - P. falciparum model, promethazine (10.0 mg/kg, once daily for 7 days) plus chloroquine (20.0 mg/kg, once daily for 7 days) only cleared the parasitemia in 1 of 2 Aotus. A similar drug regimen, but with 20.0 mg/kg of promethazine cleared parasitemia in 1 of 2 Aotus, but the infection was not cured. A second experiment, evaluated this drug combination as previously, except that during the primary treatment regimen, promethazine was administered twice daily, at 8:00 AM and 4:00 PM. A 10.0 mg/kg dose of promethazine, twice daily, for seven days plus a daily 20.0 mg/kg dose of chloroquine, cleared parasitemia (without cure) in 1 of 2 Aotus. Parasitemia in each of 2 Aotus was cleared, again without cure, when the dose of promethazine was increased to 20.0 mg/kg, twice daily administered with chloroquine. The results of the primary treatments were essentially the same as in the first pilot evaluation. Accordingly, during retreatments, promethazine was administered once daily. Retreatments cleared parasitemia in 6 of 6 Aotus, but the infection was cured in only one animal after a total three treatments. For another trial, a loading dose of chloroquine (20.0 mg/kg) was given at 8:00 AM and promethazine administered at 4:00 PM, for seven days. Primary treatment with promethazine (WR 2158AJ) at doses of 10.0 and 20.0 mg/kg plus chloroquine only suppressed parasitemias in a total of four Aotus. Retreatments with 20.0 mg/kg doses of promethazine to those animals originally administered 10.0 mg/kg doses, and 40.0 mg/kg doses of promethazine to monkeys originally administered 20.0 mg/kg doses, cleared parasitemias in the four monkeys, the infection cured in one animal.

c. WR 002173AL (BN: BK 20886), chlorpromazine
WR 006379AF (BN: BM 01907), prochlorperazine

In studies previously reported, each of these phenothiazines administered at a dose of 20.0 mg/kg once daily for 7 days with chloroquine (20.0 mg/kg x 7 days) during the ascending phase of the parasitemia reversed chloroquine resistance in vivo as shown by parasite clearance and infection cure. An important lacuna in those experiments was that the antimalarial activity, or lack thereof, for each of the drugs alone was not determined. Two experiments were initiated to obtain such essential data.

Each of two Aotus, infected with the chloroquine-resistant Vietnam Smith/RE strain of P. falciparum, received chlorpromazine, 20.0 mg/kg, once daily for 7 days. This dose had no effect upon the parasitemia, nor did administration of 2 x the original dose as both animals succumbed to a fulminating infection. The resistance to chloroquine of this strain was re-confirmed by the lack of parasite response to the maximum daily tolerated dose of chloroquine - 20.0 mg/kg.

Prochlorperazine alone (20.0 mg/kg, once daily x 7 days) had no effect upon the ascending phase of Vietnam Smith/RE parasitemia. Retreatment with 40.0 mg/kg of the drug had no activity in one Aotus and suppressed parasitemia in one animal which died of malaria.

Again, chloroquine alone possesed no antimalarial activity.

d. WR 149577AC (BM 17594), tetrandrine
WR 1544BM (AR 20613), chloroquine

In a previous study to reverse chloroquine resistance in vivo, co-administration of tetrandrine (15.0 mg/kg/7 days) and chloroquine (20.0 mg/kg/7 days) cleared Vietnam Smith/RE parasitemias in 3 of 3 Aotus, but did not cure infections. Retreatment with tetrandrine (30.0 mg/kg/7 days) and chloroquine (20.0 mg/kg/7 days) cleared parasitemia in each of two monkeys; the infection in one monkey recrudesced, and the infection in the other animal was cured. The desideratum for in vivo reversal of chloroquine resistance is infection cure following primary treatment since cure after retreatment is a combination of drug activity plus acquired immunity.

Primary treatment with tetrandrine (30.0 and 60.0 mg/kg/7 days) plus chloroquine (20.0 mg/kg x 7 days) cleared parasitemia with recrudescence in two Aotus. Retreatment with twice the respective dose of tetrandrine administered during the primary treatment plus the daily maximum tolerated dose of chloroquine (20.0 mg/kg) cleared parasitemia, but without cure.

Considering the possibility that a 7 day course of treatment was not sufficient to cure infections, the two monkeys were administered tetrandrine (25.0 mg/kg) plus chloroquine (20.0 mg/kg) for 14 days. This was the third drug regimen each monkey had received. Blood films in one Aotus were parasite negative at the time treatment was initiated. No recrudescence was observed during the post treatment observation period.

2. Bisquinoline

WR 268668AC (BN:BM 10586)

This bisquinoline was demonstrated in other laboratories to possess antimalarial activity against chloroquine-resistant strains of P. falciparum, both in vitro and in the rodent malaria model. This water insoluble drug, administered as a suspension in 0.3% methyl cellulose, had no activity or suppressive activity at doses of 1.0, 4.0 and 16.0 mg/kg, once daily, for three days, against Vietnam Smith/RE primary parasitemias. Retreatment with a dose of 4.0 mg/kg had no effect upon parasitemia in 2 of 2 Aotus, a dose of 16.0 mg/kg cleared parasitemia (without cure) in 1 of 2 Aotus, and a dose of 64.0 mg/kg cleared parasitemia in 3 of 5 monkeys, curing the infection in one subject.

Since the water insoluble form was inactive, a water soluble methylsulfonate salt was formulated and subsequently evaluated in the monkey model. Initial treatments were by the oral route and retreatments administered intramuscularly. Initial oral administration of the drug at doses of 2.0, 8.0, and 32.0 mg/kg (x 3 days) only suppressed parasitemias.

Because of the ineffectiveness of the drug by the oral route, retreatments were administered intramuscularly, the drug being dissolved in 5% dextrose solution. While infections were cured in two monkeys administered a dose of 16.0 mg/kg, this route of drug injection of doses of 32.0 and 64.0 mg/kg produced severe muscle abcesses at the site and four animals died of pathogenic sequelae.

3. Trioxanes

WR 279137AA(BN:BM 12115), trioxane
WR 279138AA(BN:BM 12124), trioxane
WR 148999AC(BN:BM 11681), tetroxane

These newly synthesized drugs were highly active in vitro and in the mouse malaria model and submitted for pilot evaluation against infections of the Vietnam Smith/RE strain. All drugs were dissolved in sesame oil and administered intramuscularly, 3 doses, at 12 hr intervals, 8:00AM, 8:00PM, and 8:00AM.

Prior to initiation of the pilot evaluation, a toxicity evaluation of

WR 148999AC used a malaria-cured Aotus, administered three 144.0 mg/kg doses. No overt adverse reactions were observed. There was no body weight loss, indicating the monkey tolerated this drug dose.

WR 279137 at a dose of 12.0 mg/kg (x3) cured 1 of 2 infections and 2 of 2 at a dose of 48.0 mg/kg (x3). WR 279138 (12.0 mg/kg x 3) did not clear primary parasitemias, but at 48.0 mg/kg cured 2 of 2 infections at primary treatment and the two treatment failures. WR 148999 (32.0 mg/kgx3) cured 2 of 2 infections and 1 of 2 infections at a dose of 144.0 mg/kg (x3).

Arteether (WR 255131AE; BL 48816) was included in this pilot evaluation as a positive drug control. A dose of 48.0 mg/kg (x3) cured the infections in each of two monkeys.

4. Antibiotics

WR 279377AC (BM 16640), azithromycin
WR 100553AA (ZM 33452), doxycycline

Among the antibacterial antibiotics, both tetracycline and doxycycline are effective against drug resistant P. falciparum infections. Although erythromycin is inactive against chloroquine-resistant falciparum infections, an analogue, azithromycin, is effective in vitro against P. falciparum and against P. berghei in the mouse model. The study reported here compares the activities of WR 279377, azithromycin and WR 100553, doxycline against infections of the multi drug resistant Vietnam Smith/RE strain of P. falciparum.

A 30.0 mg/kg dose of azithromycin administered for 7 days cleared parasitemia (with recrudescence) in one Aotus while parasitemia was only suppressed in another animal. The same primary regimen of doxycycline cleared parasitemia in each of two monkeys, but did not cure infection, although this regimen against azithromycin treatment failures did cure infections. Primary treatment with azithromycin at a 100.0 mg/kg dose for 7 days cured infections in 2 of 2 Aotus.

5. Reversal of toxicity

WR 99210AD (AW 23628)
WR 139004AC (BK 64208), folinic acid

The objective of this experiment was to determine if the toxicity of

WR 99210 could be obviated by the co-administration of folinic acid and still retain its antimalarial activity against infections of the multi-drug resistant Smith/RE strain of P. falciparum.

WR 99210 administered alone, cleared parasitemia in 2 of 2 Aotus but with recrudescence. The same regimen of WR 99210 but with 1.0 mg/kg/7 days of folinic acid cleared parasitemias in each of two monkeys, curing the infection in one. Retreatments cured 2 of 3 infections.

6. Pyrimethamine analogues

WR 250417AG (BN 34278)
WR 169626AC (BK 09350)

While these drugs, both pyrimethamine analogues have shown some activity against a pyrimethamine-resistant strain (Smith/RE) of P. falciparum in Aotus, there was some indication that resistance was induced by repeated retreatments. This present experiment was designed to determine specifically if resistance could be generated rapidly. For each drug, two infected animals each were administered a subcurative (or suppressive) dose and two infected monkeys administered a putative curative dose. Following administration of the lowest dose, if parasite suppression, or clearance with recrudescence occurred, parasites were subinoculated into a malaria naive Aotus and both donor and recipient treated with the next highest dose. If treatment failure occurred, then a second subinoculation was done, with donor and recipient being administered the putative curative dose. Drugs were given intramuscularly to diminish drug utilization and obviate any absorption problems.

Subinoculation lines and treatment with increased doses of WR 250417 are shown in Figure 1. Serial retreatments ending with a dose of 20.0 mg/kg (x3) did not induce resistance to this drug.

Similar results with WR 169626 are shown in Figure 2 in that resistance was not induced.

7. 9-phenanthrenemethanols

WR 171669AU (BM 01792), halofantrine
WR 178460AC (BM 08577), desbutylhalofantrine

Halofantrine was shown by Schmidt (16) cure Vietnam-Oak Knoll infections in Colombian Aotus when administered at a dose of 20.0 mg/kg (x7 days). The drug was less effective against Smith strain infections in Aotus of Panamanian origin. Clinical trials with halofantrine indicate its tendency to cause prolongation of QT intervals, as well as to adversely affect food intake, producing thiamine deficiency, and involve a risk of drug-drug interactions.

Desbutylhalofantrine, the metabolite of halofantrine, not only has less propensity to prolong QT intervals, but has similar or greater antimalarial activity both in vitro and in the mouse model. An experiment was designed to: 1) compare the blood schizontocidal of the two drugs against Vietnam-Oak Knoll strain infections in Panamanian Aotus, and 2) obtain blood samples for pharmacokinetic studies, using an HPLC assay.

A total of 15 Panamanian Aotus was inoculated with the Vietnam-Oak Knoll strain of P. falciparum, 6 to be treated with halofantrine, 6 to be treated with its metabolite, and 3 controls. Halofantrine, at doses of 5.0 and 20.0 mg/kg (x3) only cleared primary parasitemias. Infection cure was achieved after one or more retreatments at higher doses - 20.0, 45.0 and 90.0 mg/kg (x3).

In contrast, desbutylhalofantrine, 5.0 mg/kg, did not clear parasitemias in 3 of 3 animals but did at a dose of 20.0 mg/kg. Again, infection cure was seen after one or more retreatments, except that no cure in each of two animals following 90.0 mg/kg.

8. Pyrroloquinazoline - toxicity evaluation.

WR 227825AD (BH 35430)

This drug was effective in the murine malaria model. Before initiating antimalarial studies in the Aotus - falciparum model, the overt toxicity of WR 227825 was examined in monkeys cured of malaria infections as follows:

NM 12625 4.0 mg/kg, (oral), twice daily for 3 days. The animal died 6 days after termination of treatment, exhibiting anorexia, dehydration, and a 19% loss of bodyweight.

NM 11614 (splenectomized)

NM 12228

Each of these animals was administered an oral dose of 1.0 mg/kg, twice daily, for 3 days. NM 11614 died on day 6 post treatment, with a 21% body weight loss. NM 12228 also died on the 6th day after treatment, with a 20% loss of body weight.

The next experiment in this series further reduced the drug dose, administered to one monkey, and in a second monkey, the experimental drug was co-administered with WR 139004AC (BK 64208), folinic acid, in an attempt to prevent toxicity.

11425 WR 227825 0.1 mg/kg (oral), once daily for 3 days

12531 WR 227825 as above plus folinic acid 1.0 mg/kg (oral), once daily for 3 days.

Both animals survived without significant weight loss. Since a total dose of 0.3 mg/kg of WR 2278825 was not toxic, then it remains to be proven if folinic acid will obviate toxicity when co-administered at a known toxic dose of the pyrroloquinazoline.

Since the results of co-administration of folinic acid were inconclusive, a further study was initiated, using four Aotus cured of P. falciparum and P. vivax infections. Two animals were administered WR 227825 at a dose of 1.0 mg/kg, orally, once daily for 3 days, and 2 animals received the drug plus folinic acid, 1.0 mg/kg, once daily for 3 days. The two monkeys, with WR 227825 alone, survived, experiencing a 3% body weight loss on day 12 post treatment and a 20% body weight loss on day 6 post treatment, respectively. Both animals that received the combination regimen died, one on day 5 post treatment, 15% bodyweight loss, with gross findings of nephritis and hepatic lesions; the second monkey succumbed on day 7, post treatment, 17% body-weight loss, and showing pneumonic foci.

9. Plasmodium vivax - adaptation and treatment

a. Singleton strain

The identification of P. vivax strains less susceptible to or resistant to previously effective chloroquine regimens prompted the following study.

A patient, infected with a P. vivax infection acquired in Panama, received a putative curative regimen of chloroquine and primaquine. A relapse occurred, curative treatment given again, which was followed by a second relapse and treatment. During the first relapse, infected blood was inoculated into an Aotus, previously cured of a P. falciparum infection. The Singleton strain of P. vivax was adapted to Aotus by serial blood passage, and at the seventh passage, an experiment initiated to test the response of these parasites to chloroquine.

As anticipated, oral administration of chloroquine, WR 1544BM (AR 20613), at a dose of 1.25 mg/kg (x7) did not cure infection in 2 of 2 monkeys, but doses of 2.5 and 5.0 mg/kg (x7) cured infection in each of two Aotus, respectively.

b. AMRU-1 and AMRU-2 strains.

A cryopreserved sample of two strains of P. vivax were received from LTC G. Dennis Shanks, Army Malaria Research Unit, Ingleburn, Australia: New Guinea AMRU-1 (chloroquine resistant, from the 10th Aotus passage) and New Guinea AMRU-2 (chloroquine sensitive, 1st Aotus passage). These parasite strains were to be adapted to Panamanian Aotus, infection parameters characterized, confirm their response to chloroquine, and then expand the evaluation of WR 238605, a primaquine analogue against infections.

Each cryopreserved sample was thawed rapidly under cold, running tap water and inoculated intraperitoneally into a splenectomized monkey. All monkeys used for P. vivax studies are cured of P. falciparum infection; a patent infection of the AMRU-1 (CQR) strain began on day 15 post inoculation, while parasites of the AMRU-2 (CQS) strain were first detected on day 13 post inoculation. Parasites of each strain were then subinoculated into a second splenectomized Aotus.

Although AMRU-2 parasites developed in splenectomized Aotus, it was not possible to adapt them to unaltered hosts. An inoculum of $65x10^6$ parasites was completely ineffective in producing a patent infection.

In contrast, the AMRU-1 (CQR) strain adapted readily to normal monkeys subsequent to the second passage in a splenectomized animal. A standard inoculum of $5x10^6$ parasites produced reproducible infections. At the 13th serial passage, an experiment was initiated to confirm chloroquine resistance of the AMRU-1 strain of P. vivax.

RIII chloroquine resistance was confirmed, as total chloroquine doses o f 17.5, 35.0, and 30.0, whether administered over 7 or 3 days produced either no parasite response or transient suppression.

Evaluation of WR 238605, a primaquine analog, at the Army Medical Research Unit, Ingleburn, Australia, showed that a dose of 3 mg/kg x 3 days cured infections in 2 of 3 Aotus, and that a dose of 12 mg/kg x 3 days cured infections in 3 of 3 Aotus. An experiment was designed to confirm and expand the activity of these doses. A dose of 1.0 mg/kg x 3 days cleared parasitemias, but with recrudescence. A dose of 3.0 mg/kg x 3 days, administered during the ascending parasitemia and against recrudescences cured infections. The highest dose, 12.0 mg/kg x 3 days, as a primary treatment, cured 3 of 3 infections.

Having determined that the AMRU-1 strain of P. vivax is resistant to 10.0 mg/kg (x3) of WR 1544 (chloroquine), and that WR 238605 (a primaquine analog) at a dose of 1.0 mg/kg (x3) days will clear parasitemias, but not cure blood-induced infections, a further study was initiated to:

1. Test parasite response to the daily maximum tolerated dose of chloroquine, 20.0 mg/kg.

2. Evaluate WR 238605 at doses lower than 1.0 mg/kg to identify a suppressive only dose.

3. To evaluate WR 2975 (primaquine) against the chloroquine-resistant AMRU-1 strain.

Chloroquine (WR 1544), administered at 20.0 mg/kg (x3) cleared parasitemias with recrudescence in 2 Aotus, and only suppressed parasitemia in one subject.

Primary treatment with WR 238605AJ (BM 12562) at doses of 0.11 and 0.33 mg/kg (x3) had either no effect or a suppressive effect on parasitemia. Primary treatment with this primaquine analog at 1.0 mg/kg (3 days) cleared parasites, without cure, in 3 of 3 Aotus, while retreatment at this dose cured 4 of 5 infections. A dose of 3.0 mg/kg (x 3 days) cured 4 of 4 infections in retreated monkeys.

Primaquine, administered as a primary treatment, at doses ranging from 0.33 to 90.0 mg/kg (x3) was first effective at a dose of 10.0 mg/kg (x3), clearing parasitemia but without cure. A primary dose of 30.0 mg/kg (x3)

cured infection in 2 of 3 monkeys.

The results of this experiment show that: 1) the maximum tolerated dose (20.0 mg/kg x 3 days) cleared AMRU-1 (chloroquine resistant parasites, with recrudescence; 2) at a dose of 1.0 mg/kg (x 3 days), WR 238605 only clears infections with this parasite strain; 3) primaquine will clear these P. vivax parasites, at a dose of 10.0 mg/kg (x 3 days), in contrast with WR 238605 which clears only at 1.0 mg/kg (x 3 days).

Based upon the demonstration of the in vivo reversal of P. falciparum chloroquine-resistance, we initiated a similar experiment using the chloroquine resistant AMRU-1 strain of P. vivax, chloroquine being administered with either WR 2975 (primaquine) or WR 238605. The initial treatment doses of the two 8-aminoquinolines were selected from results of the preceeding study, while the non-effective 10.0 mg/kg (x 3 days) dose of chloroquine was used.

WR 238605, administered alone, at doses of 0.1 and 0.3 mg/kg (x 3 days), had no antimalarial activity. A dose of 1.0 mg/kg (x3 days) again cleared parasitemia, with recrudescence. Parasitemia suppression occurred when WR 238605 at a dose of 0.1 mg/kg (x3 days) plus chloroquine was administered as primary treatment and when treatment failures following 0.1 mg/kg (x3) were retreated with this dose plus chloroquine.

In contrast to no parasitemia response to a dose of 0.3 mg/kg (x3) of WR 238605 administered during the ascending phase, this dose plus chloroquine cleared (with recrudescence) parasitemias, as did retreatment with the drug combination. Moreover, a single retreatment cured the infection in 1 of 3 monkeys.

Although 1.0 mg/kg (x3) of WR 238605, alone, has proven to be non curative, this dose plus chloroquine cured infection in 2 of 3 Aotus when administered as the primary treatment. While difficult to separate from in-vivo reversal of chloroquine-resistance and acquired immunity, infections in 12 of 12 Aotus were cured after combined drug retreatment with WR 238605 (1.0 mg/kg x 3 days) plus chloroquine.

There was no evidence of chloroquine-resistance reversal using primaquine-chloroquine, whereas the WR 238605 - chloroquine combination did indicate that such reversal occurred, when the two drugs were administered at doses previously shown to be non-curative.

B. Vaccine

1. Immunogenicity of a DNA vaccine

Using Aotus cured of both P. falciparum and P. vivax infections, a series of experiments dealt with determining the optimal dose, route of delivery, and schedule for a DNA plasmid which encoded the P. yoelli CSP gene. This gene was selected because of its known immunogenicity in mice. In the first experiment with 12 Aotus, the CSP plasmid was injected intramuscularly at doses of 5, 50, and 500µg of DNA at four week intervals. Sera samples were obtained and immunofluorescence (IFA) assays performed on P. yoelli sporozoites to determine if antibodies were produced to the CSP protein. Few or no antibodies were detected by IFA.

For the second experiment, the dose was increased, the interval between doses was shortened, and the plasmid injected intramuscularly and intradermally. In some animals, the site of intramuscular injection was pretreated with bupivacaine. A total of 36 monkeys was incorporated into this experiment. Significant antibody titers (as high as 1:2560) were achieved only in the monkeys injected intradermally, without pretreatment. The dose of DNA ranged from 125 to 2000µg. These results not only demonstrated the feasibility of producing antibodies in Aotus by a DNA plasmid vaccine, but identified the intradermal route as the site of choice.

In a subsequent experiment, the number of injections sites (1, 2, and 6) to deliver the same amount of antigen were compared. Antibody titers were the highest in monkeys that had been injected six times. Following three immunizations, antibody titers in the groups vaccinated intradermally peaked briefly at week 9, but declined to 50% of their peak values by week 14. There was a general trend towards a dose response in these monkeys. By week 46, anti - Py CSP antibody titers declined to 20% and 6% of the week 14 peak values for the 2000, 500, and 125µg doses respectively. At week 47, 16 monkeys received a fourth intramuscular dose of vaccine, 8 muscle pretreated; 7 monkeys received a fourth intradermal dose. At week 49, anti - Py CSP antibody titers in the intradermally immunized groups had geometric IFAT titers of 28,963, 10,240, and 6,451 for the 2000, 500, and 125µg doses of plasmid DNA, respectively. These antibody titers were equivalent to titers generated with a Py CSP multiple Ag peptide (MAP) vaccine delivered with an adjuvant. No

significant antibody titers were detected after the fourth dose in the intramuscularly immunized groups.

2. Production of Interferon in Aotus by vaccination with Interleukin-12

Results of an experiment at another laboratory showed that when rhesus monkeys (Macaca mulatta) received rHuiL-12 on each of two days prior to inoculation with P. knowlesi sporozoites, the animals were completely protected against infection. A pilot experiment was carried out to determine if IL-12, a human agent, would stimulate gamma interferon in Aotus, as determined by serum biossay. If such stimulation occurred, an experiment was planned to ascertain if Aotus vaccinated with IL-12 would be protected when challenged with P. falciparum sporozoites.

Four Aotus, cured of P. falciparum and P. vivax infections were divided into two groups of two animals each. Group 1. Animals were vaccinated subcutaneously with 10 µg/kg of IL-12, while animals in Group 2 received 1.0 ml of 1% normal monkey serum/phosphate buffer saline. All animals were vaccinated on two consecutive days, and serum obtained approximately 72 hours after the last injection. Biossay results indicated that gamma interferon was not stimulated in either animal in Group 1.

3. Establishment of the Plasmodium falciparum (FVO strain) trophozoite model.

Of the various P. falciparum strains adapted to non-human primates, the FVO (Vietnam Oak Knoll) strain would be useful for vaccine studies as only 25-30% of infected Panamanian Aotus self-cure (3). The rest of the infected animals require curative drug treatment or death will ensue. When evaluating a vaccine, the higher the proportion of self-cure, the greater the number of animals needed in each experimental group to assure that the animals are protected by the vaccine and not self curing.

To compare the efficacy of an "artificial" vaccine with protection afforded by acquired immunity, an experiment was initiated to induce immunity by repeated trophozoite challenge. Briefly, malaria naive Panamanian Aotus were inoculated with 10^6 parasites of the FVO strain, the parasitemia monitored daily by blood film examination, and the infection cured with mefloquine (40.0 mg/kg, oral, x 3 days) when parasitemia approximated 800,000 per cmm. About 4 to 6 weeks after

infection cure, the animals were rechallenged with parasites from a donor monkey whose infection was initiated by cryopreserved parasites. Donor animals, cured of infection, were recycled into the challenge group. Challenges will be repeated until the monkeys demonstrate complete immunity.

While this is an ongoing study, the results to date summarized in Table 1, show that no monkeys were protected at the first rechallenge, 13% protected by the absence of a patent infection following the second rechallenge, and 60% protected at the third rechallenge. The fourth and fifth rechallenges indicated complete protection. Eventually, all such protected monkeys will be challenged with a heterologous strain of P. falciparum.

4. Assessment of an erythrocytic DNA vaccine - MSP-1

For the first trial of an erythrocytic vaccine in this model, a total of 9 malaria naive Aotus were divided into three groups of three animals each and vaccinated as follows:

Group 1 - gd P*f* MSP1-19	500µg ID
Group 2 - gd P*f*s 25	500µg ID
Group 3 - Vi P*f*s 25	500µg IF

Group 1 vaccine is targeted against merozoite surface protein, the Group 2 vaccine against sexual stages, and Group 3, also targeted against the sexual stages but with a different vector than in Group 2. Animals received three vaccinations at three-week intervals, and on day 14 after the last vaccination, each monkey was inoculated intravenously with 10,000 parasites of the Vietnam-Oak Knoll strain of P. falciparum.

That no protection was afforded by the vaccine is indicated by patent, virulent infections beginning on day 8 post challenge in all monkeys. Infections were cured by mefloquine treatment (40.0 mg/kg x 3 days). These monkeys were then incorporated into the Vietnam-Oak Knoll rechallenge study.

5. Establishment of the Plasmodium falciparum (Santa Lucia strain) sporozoite model

In order to test a projected plasmid DNA vaccine against Plasmodium falciparum sporozoites, it is necessary to establish a Panamanian Aotus model. The Santa Lucia strain of P. falciparum was selected because of extensive use of this parasite by Dr. W. Collins, CDC, Atlanta, GA, who has consistently obtained infections induced by sporozoites, albeit in splenectomized Aotus of South American origin. Prior to sporozoite inoculation, a sine qua non of this study was to ascertain if Panamanian Aotus would support throphozoite-induced infections of the Santa Lucia strain.

Approximately, 93 x 10^6 stage parasites were inoculated intravenously into each of two Panamanian Aotus as follows:

12732 (splenectomized) - parasites were detectable on a thick blood film on day 1 post-inoculation, with a patent period of 34 days; the maximum parasitemia of 197,120 per cmm occurred on patent day 26.
12744 (normal) - parasites were first detected on day 6 post-inoculation, followed by a patent period of 13 days, maximum parasitemia of 270 per cmm in patent day 6; after a subpatent period of 23 days, there were 13 days of patency, and a maximum parasitemia of 940 per cmm on patent day 7.

Since data indicated that the blood stages of the Santa Lucia strain will develop in Panamanian Aotus, both normal and splenectomized, Santa Lucia sporozoites were inoculated as follows: each of 12 Aotus were inoculated intravenously with approximately 20,000 sporozoites, and divided into 3 groups of 4 animals. Group 1 subjects had been splenectomized prior to inoculation; monkeys in Group 2 were splenectomized on day 7 post inoculation, and animals in Group 3 splenectomized 38 days after sporozoite inoculation.

Table 2 shows that, to date, infections were demonstrated by blood films in 2 of 4 monkeys splenectomized prior to inoculation, in 4 of 4 monkeys splenectomized on day 7 post inoculation, and in 2 of 4 still intact animals.

6. Assessment of a pre-erythrocytic DNA vaccine - CSP/SSP2/EXP1

Since it was shown that infections developed in Panamanian Aotus following sporozoite inoculation of the Santa Lucia strain of P. falciparum, a study was initiated in September 1995, that consisted of 32

malaria naive, laboratory born Panamanian Aotus, divided into four groups of eight animals each. Group 1 animals were vaccinated intradermally with CSP/SSP2/EXP-1, circum sporozoite surface protein exported protein; the animals in Group 2 received the same vaccine, administered intramuscularly, Group 3 subjects were vaccinated intramuscularly only with SSP2, Group 4, controls, received plasmid 1020 intramuscularly . Three vaccinations, approximately one month apart, were accomplished. Although the original protocol called for sporozoite challenge (20,000 sporozoites each of the Sta. Lucia strain of P. falciparum) three weeks after the last vaccination, low antibody titers precluded following the protocol. A fourth vaccination, approximately 16 weeks after the third vaccination has been accomplished. Although sporozoite challenge was scheduled at 3 weeks following the fourth vaccination, infected mosquitoes as a source of sporozoites have not become available.

7. Infectivity of the 3D7 clone sporozoites of Plasmodium falciparum in Panamanian Aotus

Due to the unreliability of obtaining Sta. Lucia sporozoites, another source was sought. The 3D7 clone of the NFS4 strain of P. falciparum, grown in vitro, produces abundant gametocytes for infecting mosquitoes by the membrane feeding technique, producing an almost on demand supply of sporozoites.

To determine if 3D7 sporozoites will produce patent infections in Panamanian Aotus, each of four normal Aotus was inoculated intravenously with 2.7×10^6 sporozoites from Anopheles stephensi mosquitoes. All monkeys were splenectomized on day 6 post inoculation, but blood films remained negative for 36 days post inoculation.

CONCLUSIONS

Of the various drugs evaluated to determine their ability to reverse chloroquine resistance in vivo when administered with chloroquine, chlorpromazine (WR 002173) and prochlorperazine (WR 006379) were the most effective by consistently curing all infections when administered during the ascending phase of the parasitemia. Lesser activity, clearance with recrudescence, was shown by protiptylene (WR 035941), tetrandine (WR 149557), and promethazine (WR 002158).

A bisquinoline (WR 268668) only suppressed parasitemias as a water-insoluble form administered orally. The water soluble methylsulfonate salt given orally only suppressed Vietnam Smith/RE parasitemias. Retreatment, administered intramuscularly, cured infections with a 16.0 mg/kg dose, but at higher doses produced severe muscle abcesses and four animals died of pathogenic sequelae.

Three newly synthesized trioxanes (WR 279137, WR 279138, and WR 148999), proved to be as effective as arteether (WR 255131) in curing Vietnam Smith/RE infections.

In comparing two antibiotics in the Aotus model, WR 279377 (azythromycin) cured Vietnam Smith/RE infections whereas WR 100553 (doxycycline) did not.

The toxicity of WR 99210 was reversed by the co-administration of folinic acid (WR 139004) and still retained its antimalarial activity.

While two pyrimethamine analogues (WR 250417 and WR 1696261) have some activity against the pyrimethamine-resistant Vietnam-Smith/RE strain, there was an indication that repeated retreatments may have induced resistance against the two drugs. Two studies attempting to induce resistance showed that such did not occur.

Halofantrine (WR 171669), a 9-phenanthrenemethanol, cures Vietnam Oak Knoll infections when administered at a dose of 20.0 mg/kg (x 7 days). Because halofantrine in clinical trials produced adverse reactions, the metabolite of halofantrine, desbutylhalofantrine (WR 178460) was compared with WR 171669 for its blood schizontocidal activity. Halofantrine only cleared primary Oak Knoll parasitemias at doses of 5.0 and 20.0 mg/kg (x 3 days), while its metabolite did not clear at a 5.0 mg/kg

dose, but did at 20.0 mg/kg. Infection cure was achieved with both drugs at higher retreatment doses.

A pyrroloquinazoline (WR 227825) was toxic at doses of 4.0 and 1.0 mg/kg (b.i.d. x 3 days). Reduction of the dose to 0.1 mg/lkg (x 3 days) with and without the co-administration of folinic acid was not toxic.

Adaptation of the Singleton strain of P. vivax to Aotus from a patient with putative chloroquine-resistance showed that the parasites were susceptible to chloroquine.

The new Guinea - AMRU-2 strain (chloroquine sensitive) could not be adapted to Panamanian Aotus, while the AMRU-1, chloroquine resistant, was readily adapted. RIII - chloroquine resistance was confirmed, as was the activity of WR 238605 (a primaquine analogue), 1.0 mg/kg (x 3 days) cleared parasitemias but with recrudescence. However, this dose combined with chloroquine (10.0 mg/kg) cured 2 of 3 infections. As expected primaquine (WR 2975) alone or in combination with chloroquine was ineffective against P. vivax blood stages.

The immunogenicity of a DNA vaccine, PyCSP, was the highest after four vaccinations by the intradermal route.

In contrast to the challenge protection against P. knowlesi - sporozoites produced by the vaccination of Interleukin-12 in rhesus monkeys, no gamma interferon was produced in Aotus.

The FVO (Vietnam Oak Knoll) strain of P. falciparum was established in Panamanian Aotus. Multiple trophozoite re-challenges have yielded a sterile immunity.

Monkeys vaccinated with an erythrocytic DNA vaccine, MSP-1, proved to be wholly susceptible to FVO strain challenge.

Panamanian Aotus were shown to support infections with the blood stages of the Santa Lucia strain of P. falciparum. Sporozoite inoculation of this strain produced patent infections in 8/12 Aotus.

A group of 32 malaria naive Aotus were vaccinated with a pre-erythrocytic DNA-vaccine - CSP/SSP2/EXP1. Because of the unavailability of Santa Lucia sporozoites, the monkeys have yet to be challenged.

A possible alternative source of sporozoites, the 3D7 clone of the NFS4 strains of P. falciparum proved to be non-infective for Panamanian Aotus .

Any evaluations of pre-erythrocytic vaccines will require a readily available source of P. falciparum sporozoites infective for Panamanian Aotus.

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FIGURE 1

ATTEMPTS TO INDUCE RESISTANCE TO WR 250417AG(BN 34278)

12719 0.25 mg/kg - Suppressed

↓

12718 0.75 mg/kg - Suppressed
12719r 0.75 mg/kg cured

12718r 2.5 mg/kg Cured

12720 0.25 mg/kg - Suppressed

↓

12717 0.75 mg/kg - Cleared/Recrudescence

12717r 2.5 mg/kg Cured

12720r 0.75 mg/kg - Cleared/Recrudescence

↓

12701 2.5 mg/kg - Suppressed
12720rr 2.5 mg/kg Cleared/Recrudescence

12701r 10.0 mg/kg Recrudescence
12720rrr 10.0 mg/kg Cured

12701rr 20.0 mg/kg - Cured

FIGURE 2

ATTEMPTS TO INDUCE RESISTANCE TO WR 169626AC(BK 09350)

12684 0.25 mg/kg - Cleared/Recrudescence

↓

12681 0.75 mg/kg - Cleared/Recrudescence
12684r 0.75 mg/kg - Cleared/Recrudescence
1684rr 5.0 mg/kg - Cured

↓

12674 5.0 mg/kg - Cured
12681r 5.0 mg/kg - Cleared/Recrudescence
12681rr 10.0 mg/kg - Cured

12686 0.25 mg/kg - Cleared/Recrudescence

↓

12725 0.75 mg/kg - Cleared/Recrudescence
12686r 0.75 mg/kg - Cured

12725r 5.0 mg/kg Cured

TABLE 1.

Infection parameters following rechallenge with the FVO strain of *Plasmodium falciparum*

Rechallenge Number	Monkeys No. Protected/No. Inoculated (No. Dead/No. Treated)	Prepatent Period Mean (Range)	Patent Period Mean (Range)	Maximum Parasitemia x 10^3 Mean (Range)
1	0/18 (4/10)	13(9-21)	18(6-22)	150(<0.01-444)
2	2/15 (2/0)	16(11-22)	10(4-20)	27(<0.01-232)
3	3/5 (0/0)	14(4-24)	7(5-9)	<0.01(<0.01-<0.01)
4	4/4 (0/0)			
5	1/1 (0/0)			

TABLE 2

SPOROZOITE-INDUCED INFECTIONS OF THE

SANTA LUCIA STRAIN OF PLASMODIUM FALCIPARUM

IN AOTUS L. LEMURINUS

MONK. NO.	PREPATENT PD. (DAYS)	MAXIMUM PARASITEMIA PER CMM (X 10^3)
	GROUP 1 - Splenectomized prior to inoculation	
12733	23	357
12734	21	434
12736		
12737		
	GROUP 2 - Splenectomized day 7 post inoculation	
12716	21	494
12741	29 (one day only)	
12743	23	616
12753	29	296
	GROUP 3 - Splenectomized day 38 post inoculation	
12746		
	23	154
12750	Positive day 25 only	
12751	39	611

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REPLY TO
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