Bull. Org. mond. Santé
Bull. Wld Hlth Org. } 1959, **21**, 233-238

Effect of Pyrimethamine upon Sporogony and Pre-erythrocytic Schizogony of *Laverania falciparum**

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Studies have been conducted in Liberia on the effect of pyrimethamine on the sporogony and, for the first time, on the pre-erythrocytic schizogony of Laverania falciparum. *From the results reported here it is concluded that it may reasonably be assumed that a mass monthly regimen of pyrimethamine in Liberia could afford protection to the individual and to the mosquito and thus to the population at large, provided that resistance to pyrimethamine does not intervene.*

Pyrimethamine in single doses of 25 mg or 50 mg administered to gametocyte carriers was able to render gametocytes of L. falciparum *uninfective to* A. gambiae *for periods up to 28 days after administration.*

Mosquitos feeding upon a malaria-free pyrimethamine-treated subject before or after feeding upon a non-treated gametocyte carrier became infected and sporozoites appeared in the salivary glands.

Pyrimethamine administered in 12.5-mg doses 13 days before, 6 days before and 2 days after sporozoite infection or administered in 25-mg doses 35 days and 7 days before sporozoite infection disallowed the development of the pre-erythrocytic schizonts of L. falciparum *in the livers of two chimpanzees.*

The effect of pyrimethamine and other possibly active drugs upon sporogony of malaria parasites has been investigated by various authors. Findlay et al. (1946) studied the effect of sulfonamides; Fairley et al. (1946), Mackerras & Ercole (1947), Shute & Maryon (1948) and Terzian & Weathersby (1949) the effect of proguanil; and Foy & Kondi (1952), Shute & Maryon (1954), Jeffery et al. (1956) and Burgess & Young (1959) the effect of pyrimethamine on the sporogony of *Laverania falciparum*.[5]

Fairley et al. (1946), Mackerras & Ercole (1947), Shute & Maryon (1948) studied the effect of proguanil and Shute & Maryon (1954) and Young & Burgess (1959) the effect of pyrimethamine on the sporogony of *Plasmodium vivax*. Whitman (1948) investigated the effect of hydroxynaphthoquinone and Terzian, Stahler & Weathersby (1949) the effect of sulfadiazine, metachloridine and proguanil on the sporogony of *Haemamoeba gallinaceum*.

The effect of causally prophylactic antimalarial drugs upon pre-erythrocytic schizogony of mammalian malaria parasites has been investigated directly only by Hawking & Thurston (1952). They studied the effects of sulfonamides, proguanil and pamaquin upon pre-erythrocytic schizonts of *Plasmodium cynomolgi*. Indirect studies of the effect of drugs upon the tissue phase have been summarized by Bray (1957).

In only one of the inquiries (Burgess & Young, 1959) has the long-range effect of pyrimethamine upon sporogony been investigated; in none of the inquiries has the effect of this drug upon pre-erythrocytic schizogony of mammalian malaria parasites been studied directly.

* From the Liberian Institute of the American Foundation for Tropical Medicine, Inc., Harbel, Liberia. This investigation was supported in part by a research grant (RG-4533) from the National Institutes of Health, Public Health Service, US Department of Health, Education, and Welfare, and in part by a grant from the World Health Organization.

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[5] While the term *Plasmodium falciparum* is in common use, the choice between the generic names *Plasmodium* and *Laverania* is left open by the International Commission on Zoological Nomenclature (see that Commission's Opinion No. 283).

It has been proposed on the basis of studies by Miller (1957) to apply experimental mass chemotherapy of malaria in Liberia by using 25 mg or 50 mg of pyrimethamine monthly in a large population. The object of the present studies has been to investigate possible effects of such a regimen of pyrimethamine upon malaria, other than its well-known effects in the blood of the vertebrate host. Specifically, it was the intention to discover if a single monthly dose of pyrimethamine afforded any protection to the population as a whole by disallowing mosquito infections and what effect such a regimen exerted upon tissue schizonts.

MATERIALS AND METHODS

L. falciparum in indigenous Liberians in the vicinity of the Institute was used in all experiments except one, when a strain of *P. vivax schwetzi* in a chimpanzee was used.

Anopheles gambiae obtained from a laboratory colony was used in all experiments except one, when *A. melas* was used.

Gametocyte carriers were disclosed by local surveys and were brought to the laboratory, where leucocyte and gametocyte counts were made. In experiment 1 a batch of 50 or more mosquitos was then fed upon each suitable individual. Subsequently pyrimethamine (25-mg tablets) was administered orally. Further batches of 50 or more mosquitos were then fed on the carrier at suitable intervals.

All mosquitos were maintained at room temperature (74°-87°F or about 23.5°-30.5°C) on sugar solutions subsequent to the feed.

To study sporogony, mosquitos were dissected from 4 to 9 days after the blood meal to observe oocysts, and at 10 to 14 days after the blood meal to observe sporozoites. If retarded oocysts were seen, dissections were delayed up to 17 days to gauge any possible development. In an attempt to discover the exact point and the effect of pyrimethamine activity against sporogony, microgamete and ookinete formation was studied. Mosquito mid-guts two and three days after blood meals on both control and treated individuals were taken, fixed, sectioned and stained by the Giemsa-colophonium method or by Giemsa followed by acetic acid differentiation and acetone dehydration.

In experiment 2, batches of mosquitos were fed twice, one feed being on an untreated gametocyte carrier, the other on a malaria-free person who had taken pyrimethamine previously.

In experiment 3, involving the tissue stages, the vertebrate host was the chimpanzee, *Pan troglodytes verus*. Chimpanzees were infected by the intravenous inoculation of salivary glands from a single batch of *A. gambiae* infected with *L. falciparum*. Salivary glands were dissected out into 25% inactivated human serum in physiological saline. Liver biopsies were performed by open laparotomy. The liver pieces were fixed in Carnoy's fluid, sectioned and stained by the Giemsa-colophonium method.

EXPERIMENTAL PROTOCOLS

Experiment 1. Effect upon sporogony in mosquitos fed on crescent-carriers treated with single dose of 25 mg or 50 mg of pyrimethamine

The subjects were four adults and six children aged 1-10 years. The results are shown in Table 1.

It can be seen that at these dosages pyrimethamine protected the mosquito from infection with sporozoites for times varying from 1 to 28 days after drug administration. Gametocyte counts as high as 1724 gametocytes/mm^3 were recorded. Pre-drug feeds were constantly positive, mosquitos showing sporozoites in the glands. Post-drug feeds were consistently negative for sporozoites.

Some retarded oocysts were seen but these were always very small, of the size of normal 2- and 3-day-old oocysts, and did not increase in size during 11 days of observation. At 11 days they had lost the definition of a cyst and their presence could be recognized only by the small mass of remaining pigment. Their pigment pattern was always that of a normal 3-day-old oocyst.

The examination of blood smears taken from gorged mosquitos and of sections of mosquito guts revealed that microgamete formation and ookinete formation, including the presence of nuclei in microgametes, was normal when the engorgement was on either untreated or treated gametocyte carriers. Ookinetes and numerous uni-nucleated oocysts were found in gut sections 48 hours after engorgement on untreated subjects. Ookinetes but no oocysts were found in gut sections 48 hours after engorgement on treated subjects. No signs of penetration or attempted penetration of ookinetes could be found in gut sections from mosquitos gorged on treated subjects. Forty-eight hours after mosquitos had been fed on treated subjects, ookinetes showed poor stain uptake and the nuclei were invisible after acetic acid differentiation of the Giemsa stain.

TABLE 1

EFFECT OF PYRIMETHAMINE ON SPOROGONY WHEN ADMINISTERED TO THE GAMETOCYTE DONOR

Subject	Age	Pyrimethamine on day 0 (mg)	Days from drug administration to mosquito feed	Results [a]			
				Gametocytes/mm³	Number dissected	Number positive	Percentage positive
T. M.	Adult	25	—1	95	61	16	26
			3	—	*30*	*0*	*0*
J. C.	Adult	25	0	70	24	16	67
			3	*84*	*50*	*1* [b]	*2* [b]
			6	*85*	*41*	*0*	*0*
			9	*55*	*26*	*0*	*0*
			12	—	*31*	*0*	*0*
J. K.	Adult	25	0	16	20	1	5
			3	*38*	*39*	*0*	*0*
			6	*8*	*20*	*0*	*0*
B. W.	10 years	25	0	454	49	39	80
			1	*481*	*42*	*0*	*0*
			3	*276*	*36*	*0*	*0*
			6	*57*	*24*	*0*	*0*
			16	*112*	*27*	*0*	*0*
			21	*59*	*35*	*0*	*0*
			28	*17*	*42*	*0*	*0*
P. M.	9 years	25	0	180	46	36	76
			7	*123*	*32*	*0*	*0*
W. J.	7 years	25	0	636	42 [c]	35	83
			14	*292*	*77*	*8* [b]	*10* [b]
S. M.	4 years	25	0	189	51	38	74
			7	*115*	*10*	*0*	*0*
W. D.	Adult	50	0	178	35	31	89
			14	*38*	*36*	*0*	*0*
D. J.	6 years	25	0	516	18	10	56
			1	*498*	*31*	*2* [b]	*6* [b]
G. D.	1 year	25	0	1 696	14	11	79
			1	*1 724*	*17*	*0*	*0*
			28	*221*	*20*	*0*	*0*

[a] Figures in roman type refer to control feedings before drug administration; figures in italics to feeds following drug administration.

[b] Retarded oocysts

[c] *A. melas*

Experiment 2. Effect upon sporogony in mosquitos fed on pyrimethamine-treated subjects before or after an infective blood meal

The results of this experiment are shown in Tables 2-4. In the first part of the experiment (Table 2), four batches of mosquitos were fed on a human crescent-carrier and four batches on a chimpanzee having gametocytes of *P. schwetzi* in the blood. Four days later three batches from each group were fed on subjects who had received 25 mg of pyrimethamine one, two, three, four or six days earlier. Two batches, one from each group, were kept as controls.

In the second part of the experiment (Table 3), a similar experiment was done using a 50-mg dose of pyrimethamine administered one hour and 24 hours before the feed, and mosquitos (*A. melas*) were fed on the treated subject two and three days respectively after the feed on the crescent-carrier.

The results indicate that pyrimethamine taken up with a blood meal by mosquitos after an infective blood meal was unable to prevent sporozoite infection in the glands of the mosquito. There was a significant reduction in oocyst rate when 50 mg of pyrimethamine were administered to the subject one hour before the mosquitos were fed. However, sporozoites still appeared and thus for practical purposes this difference can be ignored.

TABLE 2

EFFECT UPON SPOROGONY IN *A. GAMBIAE* FED ON PYRIMETHAMINE-TREATED SUBJECTS (25-mg dose) FOUR DAYS AFTER FEEDING ON GAMETOCYTE CARRIERS

Mosquito group	Parasite species	Gametocytes/mm³	Days from drug administration to mosquito feed	Results		
				Number dissected	Number positive	Percentage positive
A	*L. falciparum*	54	—	9	3	33
B	*L. falciparum*	54	6	16	6	38
C	*L. falciparum*	54	4	13	6	46
D	*L. falciparum*	54	2	10	3	30
A	*P. v. schwetzi*	78 [a]	—	15	7	47
B	*P. v. schwetzi*	78 [a]	3	10	4	40
C	*P. v. schwetzi*	78 [a]	2	12	4	33
D	*P. v. schwetzi*	78 [a]	1	13	6	46

[a] As microgametocytes/mm³

TABLE 3

EFFECT UPON SPOROGONY IN *A.MELAS* FED ON A PYRIMETHAMINE-TREATED SUBJECT (50-mg dose) TWO AND THREE DAYS AFTER FEEDING ON A CRESCENT-CARRIER

Mosquito group	Gametocytes/mm³	Hours from drug administration to mosquito feed	Days from cresent feed to drug feed	Results		
				Number dissected	Number positive	Percentage positive
A	636	—	—	42	35	83
B	636	1	2	23	10	43
C	636	24	3	23	18	78

In the third part of the experiment (Table 4), batches of mosquitos were fed first on malaria-free subjects who had received pyrimethamine, and three days later on untreated crescent-carriers. Other batches were fed on the crescent-carriers only and served as controls.

The results indicate that allowing mosquitos to take blood from pyrimethamine-treated subjects three days before an infective blood meal had no effect on the course of sporogony.

TABLE 4

EFFECT UPON SPOROGONY IN *A. GAMBIAE* FED ON PYRIMETHAMINE-TREATED SUBJECTS (25-mg and 50-mg doses) THREE DAYS BEFORE FEEDING ON CRESCENT CARRIERS

Mosquito group	Pyrimethamine dose (mg)	Days from drug administration to mosquito feed	Gametocytes/mm³	Results		
				Number dissected	Number positive	Percentage positive
A	—	—	16	40	9	23
B	25	1, 8, 15 etc.[a]	16	33	8	24
C	—	—	189	51	38	74
D	50	1	189	49	21	43[b]

[a] Subject taking pyrimethamine weekly

[b] Much lesser uptake of blood than controls

Experiment 3. Effect of weekly and monthly pyrimethamine regimens on tissue stages of L. falciparum *in the chimpanzee*

Each of three chimpanzees (Nos. 38, 36 and 24; see Table 5) were infected by intravenous inoculation of 110 pairs of salivary glands from a single batch of mosquitos heavily infected with sporozoites of *L. falciparum*. The oocyst rate in the batch was 79% and the number of oocysts per mosquito varied from 9 to 623, average 266.

No. 38 was given no drug. No. 36 (3.2 kg) received 12.5 mg of pyrimethamine 13 days before, 6 days before and 2 days after inoculation. No. 24 (3.8 kg) received 25 mg of pyrimethamine 35 days before and 7 days before inoculation. Liver biopsies were taken from all animals 6 days after inoculation.

From Table 5 it can be seen that while the control animal showed 5300 schizonts/cm³ of liver, both treated animals failed to show parasites in approximately 0.5 cm³ of liver tissue.

TABLE 5

EFFECT OF WEEKLY AND MONTHLY REGIMENS OF PYRIMETHAMINE UPON PRE-ERYTHROCYTIC SCHIZOGONY OF *L. FALCIPARUM* IN CHIMPANZEE LIVER

Chimpanzee No.	Pyrimethamine		Infection		No. of pre-erythrocytic schizonts/cm³	Blood infection on 7th day after inoculation
	Dose (mg)	Day	No. of mosquito glands inoculated	Day		
38	—	—	110	0	5300	heavy
36	12.5	—13, —6, +2	110	0	0	0
24	25	—35, —7	110	0	0	0

Blood films showed no parasites in either treated animal. In the control animal tiny rings of *L. falciparum* were seen for the first time at 6 days and 2 hours after infection. Within six hours this rose to a very heavy parasitaemia which persisted, as rings only, through the seventh day after infection. On the eighth day parasitaemia dropped considerably but remained easily visible through the ninth day. On the tenth day only one ring was found after long search and later on the tenth day no parasites could be found. The blood then remained negative during an observation period of three months.

DISCUSSION

It would appear that the administration of a single dose of 25 mg or 50 mg of pyrimethamine to a gametocyte carrier can disallow *L. falciparum* sporozoite formation in mosquitos feeding up to 28 days after treatment of the gametocyte carrier under the conditions obtaining in Liberia. Thus as a working hypothesis it can be inferred that a monthly regimen of pyrimethamine could protect not only the individual from infection but also the community as a whole from challenge by *L. falciparum*, provided that all individuals are treated and that no pyrimethamine-resistant strains of *L. falciparum* are present.

On the other hand, if during mass chemotherapy some gametocyte carriers remain untreated, the presence of pyrimethamine in the remainder of the population gives no protection against challenge to the community, as blood meals taken by mosquitos from pyrimethamine-treated persons before or after an infective feed give no protection against eventual sporozoite infection of the mosquito. This is in accordance with the results obtained for hydroxy-naphthoquinone by Whitman (1948).

Lastly, the regimens of pyrimethamine as used afforded complete protection to the chimpanzee liver from the growth of pre-erythrocytic schizonts of *L. falciparum* under the conditions of the experiment. It is not known if protection would have been complete had the infection taken place at 20-25 days after the last drug administration in the monthly regimen.

The results of these types of experiments in Liberia have differed in certain respects from results obtained elsewhere. Firstly, it should be stated that as far as is known no pyrimethamine-resistance exists in Liberian *L. falciparum* as yet, and all results reported here refer to drug-sensitive strains. It can be said that Liberian *L. falciparum* has proved exceptionally sensitive to pyrimethamine under existing conditions of hyperendemicity.

This is obviously the case when the results of Miller (1957) in Liberia are compared with, say, those of Clyde & Shute (1954) in Tanganyika; in Liberia two years of mass treatment with pyrimethamine failed to produce resistance, whereas, in some areas in Tanganyika, resistance was produced after six months of treatment. This sensitivity is again illustrated by the results reported here when they are compared with those of Shute & Maryon (1954) and Burgess & Young (1959). Shute & Maryon (1954) studied the effect of the drug on sporogony of West African *L. falciparum* (in *A. stephensi*) up to 6 days after drug administration to non-immune white persons. In their results the production of small retarded oocysts in reasonable numbers was the rule rather than the exception. Burgess & Young (1959) studied the effect of the drug on sporogony of New World strains of *L. falciparum* (in *A. freeborni* and *A. quadrimaculatus*) up to 33 days after drug administration to presumably non-immune Negroes. Again the production of small retarded oocysts was the rule. In both cases it is stated that gametogony is unaffected, and the effect of the drug is first noted at the time of the first division of the oocyst. However, it is also obvious from the figures given in these two communications that a large proportion of ookinetes as compared with controls never penetrate the gut wall.

In the work reported here (involving *A. gambiae*) the ookinete which penetrates the gut wall to round up into an oocyst is the exception, not the rule. The greatest part of the effect of the drug is in fact to prevent the ookinete from penetrating the gut wall, and thus discussion in this case of the effect of pyrimethamine upon the first or second division of the oocyst (whichever is the first asexual schizogonic division) is irrelevant. Though it is known that pyrimethamine is a specific folic acid antagonist and that folic acid is an apparent essential to schizogonic division of malaria parasites, this is of no assistance in the present case as sporogonic (schizogonic) division is not involved.

What can cause the ookinete to fail to penetrate the gut wall remains a mystery. In the present experiments in blood from pyrimethamine-treated subjects the microgametocytes produced nucleated microgametes, ookinetes were formed in as large a number as in controls but sections of mosquito guts showed that whereas the ookinetes in controls penetrated the gut, no attempt at gut penetration could be found after pyrimethamine treatment —though penetration can occur, as evidenced by the retarded oocysts sometimes seen on mid-guts. In some ookinetes, 22 hours after the infected feed by the mosquito, an occasional large piece of extra-nuclear chromatin led to the suspicion that the male and female nuclei had not fused and the zygote had not in fact been formed. It is possible that this may cause loss of co-ordination and organization of the parasite so that gut penetration becomes a matter of chance, not direction. On the other hand, the extra-nuclear chromatin seen could as easily have been a large "chromidium" and have no significance.

ACKNOWLEDGEMENTS

We wish to thank Dr A. E. Gunders for his assistance in the dissection of mosquitos and the laparotomy of chimpanzees and Mr John Freeman for the preparation of all histological specimens.

RÉSUMÉ

On a étudié au Liberia l'effet de la pyriméthamine sur la sporogonie de *Laverania falciparum* et, pour la première fois, sur sa schizogonie pré-érythrocytaire. D'après les résultats de ces études, il est raisonnable de supposer qu'on peut protéger l'individu et le moustique — par conséquent toute la population de ce pays — en appliquant systématiquement un schéma posologique mensuel. Il ne faudrait évidemment pas qu'une résistance au médicament survienne.

Administrée à des porteurs de gamétocytes en doses uniques de 25 mg ou de 50 mg, la pyriméthamine rend les gamétocytes de *L. falciparum* non infectants pour *A. gambiae* pendant des périodes allant jusqu'à 28 jours après l'administration du produit.

Des moustiques qui, avant ou après avoir piqué un porteur de gamétocytes non traité, se sont nourris sur un sujet non impaludé mais traité à la pyriméthamine, ont été infectés. La pyriméthamine n'a donc pas empêché l'apparition des sporozoïtes dans leurs glandes salivaires.

La pyriméthamine, en doses de 12,5 mg administrées 13 jours et 6 jours avant l'infection par le sporozoïte et 2 jours après, ou en doses de 25 mg administrées 35 jours et 7 jours avant l'infection, a empêché le développement des schizontes pré-érythrocytaires de *L. falciparum* dans le foie de deux chimpanzés.

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