THE SELECTIVE ACTION OF SULFANILAMIDE ON THE PARASITES OF EXPERIMENTAL MALARIA IN MONKEYS IN VIVO AND IN VITRO

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PLATE 1

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Previous work has shown that the administration of as little as 1 gm. of sulfanilamide by mouth will completely eradicate both acute and chronic *Plasmodium knowlesi* infections in *rhesus* monkeys (1). The evidence of cure in these cases was: (*a*) the permanent disappearance of parasites from the circulating blood; (*b*) the failure to reproduce the infection in normal monkeys by the subinoculation of fresh blood from treated animals; (*c*) the failure of treated monkeys to relapse following splenectomy; and (*d*) the successful reinfection of treated monkeys with the homologous parasite. These findings were of special interest because untreated *P. knowlesi* malaria in monkeys is an infection that is almost invariably fatal, and in this respect it represents one of the most virulent malaria infections in either human beings or experimental animals.

On the other hand, it has been the experience in this laboratory, as well as of most other workers, that sulfanilamide is an inferior therapeutic agent in human malaria (2–4). The drug has also failed to exert any demonstrable influence, even with excessive doses, in canaries and chicks infected with *Plasmodium cathemerium* and *Plasmodium lophurae*, respectively (5).

From the above mentioned findings it has been impossible to determine whether the activity of the drug varied from host to host, or whether it exerted its influence according to the species of plasmodium encountered. In order to elucidate this point, additional studies on the action of sulfanilamide on two plasmodial infections in a common host and on the effect of the drug on the metabolism of the parasites *in vitro* are here reported.

Materials

The host used was the *rhesus* monkey and the two species of parasites employed were *Plasmodium knowlesi* and *Plasmodium inui* (6).

The minimal infective dose for *P. knowlesi* is probably between one and ten parasites (7), and since there are no known insect vectors, infected blood was used for the inoculations. Infections thus established, unless interrupted early with antimalaria drugs, usually terminate in the death of the animal. At the time of death 50 per cent or more of the red blood cells contain parasites, and there is a profound anemia.

P. inui produces a moderately severe infection following the injection of infected blood. The parasite density at the height of the acute disease rarely exceeds 15 per cent of the normal red blood cells, and the acute attack usually subsides spontaneously into a chronic infection of indefinite duration.

Mixed infections in monkeys to be used for treatment were produced by injecting viable *P. knowlesi* parasites into monkeys with chronic *P. inui* infections. Mixed infections in the controls were obtained in three ways: (*a*) the simultaneous injection of the two parasites, (*b*) superinfection of monkeys harboring *P. inui* infections with *P. knowlesi* parasites, and (*c*) superinfection of monkeys with chronic *P. knowlesi* infections with *P. inui* parasites.

For treatment of the infected monkey sulfanilamide (para-amido-benzene sulfonamide) was used, and the drug was always given by mouth in 1 gm. doses.

Effect of Sulfanilamide on Simple P. knowlesi and P. inui Infections

The dramatic manner in which sulfanilamide effects a cure of *P. knowlesi* infections in *rhesus* monkeys was briefly summarized above.

Experiment 1.—In order to test its effect against *P. inui* infections, monkeys 1, 2, and 3 were injected and treated as shown in Table I. Normal monkeys 1 and 2 were each inoculated intra-abdominally with 1 cc. of parasitized fresh blood. Daily blood smear examinations revealed typical parasites in both monkeys by the 6th day. Monkey 1 was then given three daily 1 gm. doses of sulfanilamide by mouth, while monkey 2 was kept as an untreated control. During the first 48 hours of the period of treatment of monkey 1 there was a slow increase in the parasite count. This was followed by a gradual decline to a point where plasmodia were absent from the smears. This picture persisted for approximately 2 weeks, when the parasites reappeared and steadily increased in number. Monkey 2, the untreated control, had a typical *P. inui* infection with the peak of the acute attack occurring on the 14th day. 6 weeks later, with both animals having identical parasite counts, monkeys 1 and 2 were each given three daily 1 gm. doses of sulfanilamide, this being the second course of treatment for monkey 1 and the first for monkey 2. Again there was a temporary reduction in circulating parasites in both monkeys, and smears were never negative for more than a few days at a time. Monkey 3, used to repeat the experiment, had a chronic *P. inui* infection of 3 months' duration. Its parasite count was 15[1] at the time when sulfanilamide was first given. Three daily 1 gm. doses produced no significant effect on the parasite density until 48 hours after the third dose, when there was a diminution. For 3 weeks the daily smears showed only an occasional parasite from time to time, and the count then returned to its pretreatment level.

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[1] Parasite counts given in this paper refer to the number of parasitized cells per 10,000 red cells.

The outcome of these results is seen in Table I. It was observed that sulfanilamide exerted only a temporary influence on *P. inui* in *rhesus* monkeys, regardless of whether they were treated during the acute, subacute, or chronic stages of the disease. This was true even with dosage in excess of the amount found necessary to eradicate the more virulent *P. knowlesi* infections in the same species of monkey.

Effect of Sulfanilamide in Mixed Infections

To obviate the influence of such factors as individual host variation, differences in the composition of the drug used on different days, and variations in the number and stage of parasites in the inoculum, an attempt was made to demonstrate the selective activity of sulfanilamide in mixed infections in the same experimental animal. One should thus have a better opportunity to observe the effects directly or indirectly on the parasite itself.

Experiment 2.—Monkeys 4 and 5, shown in Table I, were suffering with chronic *P. inui* infection, and each was superinfected with 10,000 *P. knowlesi* parasites. When the total parasite count of monkey 4 had increased from 16 to 160, and both species of parasite were identified in the smears from morphological characteristics, the animal was given a 1 gm. dose of sulfanilamide by mouth. On the 3rd day following treatment parasites completely disappeared from the circulating blood. During this interval there was marked phagocytosis of malaria parasites by the circulating macrophages, as shown in Figs. 1 A and 1 B, some of which contained as many as ten parasitized red cells. Parasites did not reappear until 27 days after treatment, when they were identified as *P. inui* morphologically. This fact was confirmed by subinoculation into a normal monkey, which developed a typical chronic *P. inui* infection from monkey 4. Monkey 5 remained untreated until the parasite count was 2,880 and the red count was 2,100,000. The infection was predominantly *P. knowlesi*, although there were also present some of the *P. inui* variety. At this point monkey 5 was so critically ill that it was transfused with 50 cc. of citrated normal monkey blood before being given 1 gm. of sulfanilamide by mouth. On the following day about half the cells were parasitized (count 4,530), and a second transfusion of 40 cc. of normal blood was given along with 2 gm. of the drug. The parasite count dropped to 46 within 24 hours and was zero in 48 hours. During this period there was great activity of circulating macrophages in the phagocytosis of infected cells, as shown in Fig. 1 A and 1 B. Parasites outside the macrophages appeared distorted and were poorly stained. Thereafter there was an absence of parasites for 34 days, and when they reappeared they were identified morphologically as *P. inui*. This identification was confirmed, as with monkey 4, by subinoculation of blood into a normal monkey, and a resultant simple *P. inui* infection.

The results seen in Table I showed that sulfanilamide, either directly or indirectly, had the ability to remove a virulent plasmodial infection and was practically without influence on the milder *P. inui* infection in the same experimental animal.

TABLE I

Results of Sulfanilamide Therapy on Simple P. inui and Mixed P. knowlesi and P. inui Malaria Infections in Rhesus Monkeys and Untreated Controls

Monkey No.	History of previous infection: Kind	History of previous infection: Duration	Parasite used for inoculation	Treatment with sulfanilamide: Time after last inoculation at beginning of treatment	Treatment with sulfanilamide: Parasite count at beginning of treatment	Results
			Treated *P. inui* Infections			
		days		*days*		
1	Normal monkey	—	*P. inui*	5 49*	+ +	Temporary suppression of infection
2	Normal monkey	—	*P. inui*	49	12	Temporary suppression of infection
3	*P. inui*	90	—	64	32	Temporary suppression of infection
			Treated Mixed Infections			
4	*P. inui*	110	*P. knowlesi*	14	160	Disappearance of *P. knowlesi* and temporary suppression of *P. inui*
5	*P. inui*	115	*P. knowlesi*	10	2,880	Disappearance of *P. knowlesi* and temporary suppression of *P. inui*
			Untreated Mixed Infections (Controls)			
6	*P. inui*	68	*P. knowlesi*	—	—	Died 11th day of *P. knowlesi* infection
7	*P. inui*	75	*P. knowlesi*	—	—	Died 7th day of *P. knowlesi* infection
8	*P. knowlesi*	100	*P. inui*	—	—	Typical *P. inui* infection
9	*P. knowlesi*	115	*P. inui*	—	—	Typical *P. inui* infection
10	Normal monkey	—	*P. knowlesi* and *P. inui*	—	—	Died 13th day of *P. knowlesi* infection
11	Normal monkey	—	*P. knowlesi* and *P. inui*	—	—	Died 11th day of *P. knowlesi* infection

* Same monkey treated twice with sulfanilamide.

\+ indicates less than one parasite per 10,000 R.B.C.

Effect of Mixed Plasmodial Infection in Untreated Monkeys

In order to demonstrate that the drug was responsible for the disappearance of the more virulent parasite and not an antagonistic effect in mixed infections, monkeys were infected with both types of parasites and their infections allowed to go untreated.

Experiment 3.—The details and results of this experiment are summarized in Table I. Monkeys 6 and 7 with chronic *P. inui* infections of 68 and 75 days' duration were inoculated with 10,000 *P. knowlesi* parasites. Both monkeys showed the presence of a mixed infection on the 5th day after inoculation and died on the 11th and 7th days, respectively. Monkeys 8 and 9, with chronic *P. knowlesi* infections established by quinine some months previously, were similarly infected with viable *P. inui* parasites. In both instances the inoculated monkeys experienced a typical mild *P. inui* infection. Normal monkeys 10 and 11 were each inoculated with a mixture of 10,000,000 *P. inui* and 10,000 *P. knowlesi* parasites. Both monkeys had mixed infections on the 7th day and died on the 13th and 11th day, respectively, the *P. knowlesi* parasites having multiplied at their usual rate overshadowing the mild infections.

The results of these controls, summarized in Table I, show that in untreated mixed infections there is no evidence of an antagonistic effect between the two plasmodia.

The Effect of Sulfanilamide on the Metabolism of the Parasites in Vitro

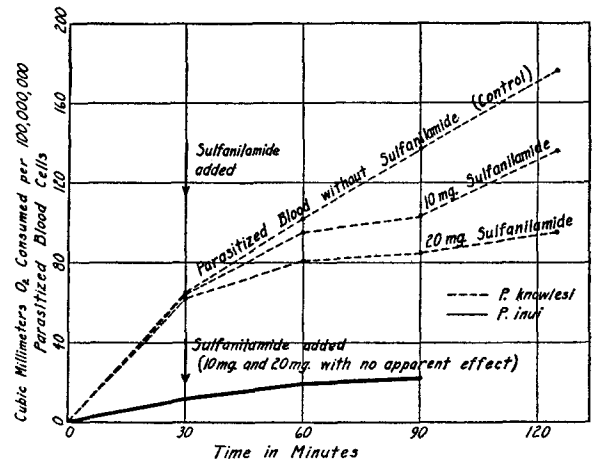
The difference in the reaction of plasmodial infections to sulfanilamide in a common host was taken as evidence that the drug acted directly on the parasite independently of the host. An attempt was made to find whether the same difference would make itself manifest when sulfanilamide was added to the parasites *in vitro*. This was done by testing the effect of sulfanilamide on the respiration of the two parasites in a Warburg manometer.[2]

The parasites were obtained by bleeding monkeys with well advanced infections. In order to obtain *P. inui* infections that were comparable in intensity to *P. knowlesi*, it was necessary to use splenectomized monkeys. Heparinized infected blood was centrifuged and the brown parasite substance, which has a lower density, was removed from the top layer of the cellular material. This was then resuspended in the same monkey's serum in the ratio of one part of parasites to three parts of serum. The number of reticulocytes and leukocytes in the mixture was below the concentration at which their metabolism could interfere with the results. The system used was essentially that of Christophers and Fulton (8), with the exception that glucose was added to the medium. The sulfanilamide was put in a side arm with Ringer solution at the end of a 30 minute control run. To absorb the CO_2 produced, 0.3 cc. of 40 per cent KOH was put in the center cup. Glycolysis was run in the same medium with $NaHCO_3$ added.

The results as shown in Text-fig. 1 are based on 100 million parasites, and it was found that the oxygen consumption for *P. knowlesi* was ap-

[2] The determinations on the metabolic rate of the parasites were done by Mr. Charles Kensler through the courtesy and in the laboratories of Dr. C. P. Rhoads at The Rockefeller Institute for Medical Research.

proximately six times greater than for the same number of *P. inui* parasites. Although the concentration of sulfanilamide (20 mg. in system) was approximately one-third that used to cure a monkey, there was a marked paralyzing effect on the respiration of the parasite. With 10 mg. of sulfanilamide the effect was definite, but to a less degree. There was no observable effect on the *P. inui* parasites by the same concentrations of the drug. The anaerobic CO_2 production of both parasites was unaffected by sulfanilamide. The R.Q. of *P. knowlesi*, confirming Christophers and Fulton (8), and of *P. inui* was found to be slightly less than one.



TEXT-FIG. 1. Influence of sulfanilamide on the rate of oxygen consumption of *P. knowlesi* and *P. inui* parasitized red cells *in vitro*.

Effect of Sulfanilamide against P. knowlesi in Other Hosts

The next point taken up was whether sulfanilamide is effective against the parasites regardless of the host. Human volunteers with general paresis and resistant to therapeutic *vivax* malaria were inoculated with *P. knowlesi* infected blood and subsequently treated with the drug. Although the infections were mild, they disappeared promptly after the administration of sulfanilamide, but the extremely variable character of untreated *P. knowlesi* infections in man does not allow for more than inferential evidence of the action of the drug.

Two *cynomolgos* monkeys were inoculated with blood infected with *P. knowlesi*, and in this instance also the resultant infection in untreated controls was so mild and transitory that no definite conclusions can be drawn.

DISCUSSION

The highly selective action of sulfanilamide against two species of plasmodia in the same experimental animal and the parallel effect when tested on the metabolism of the parasites *in vitro* offer several points for discussion. They indicate the necessity of taking into consideration the metabolism of parasites themselves as a factor when testing the effectiveness of any antimalaria drug. No therapeutic agent should be discarded on the basis of a negative result against one plasmodium, nor can such an agent be proclaimed as a universal remedy, regardless of its effectiveness against one type of infection.

No attempt was made in this study to learn why sulfanilamide has an inhibitory action upon one malaria parasite and not upon another closely related one. Results obtained from numerous studies of its action on bacteria suggest that the effect of the drug is probably an oxidation process resulting either in partial or complete interference with the metabolism of the parasite. The results of this study confirm the suggestion made by Fulton and Christophers (9) that the influence of an antimalaria drug on the metabolism of a malaria parasite *in vitro* gives the most direct indication regarding its efficiency as a therapeutic agent.

SUMMARY

It was found that with mixed malaria infections in the same experimental animal sulfanilamide eradicates a virulent *P. knowlesi* infection, leaving the animal with a milder chronic *P. inui* infection. Determinations of the metabolic activity of the two parasites *in vitro* showed that *P. knowlesi* consumed approximately six times more oxygen than *P. inui*. The addition of sulfanilamide in concentrations less than that necessary to effect a cure almost completely inhibited the respiration of *P. knowlesi* parasites *in vitro*, while the same concentrations of the drug against the same number of *P. inui* parasites had no apparent effect.

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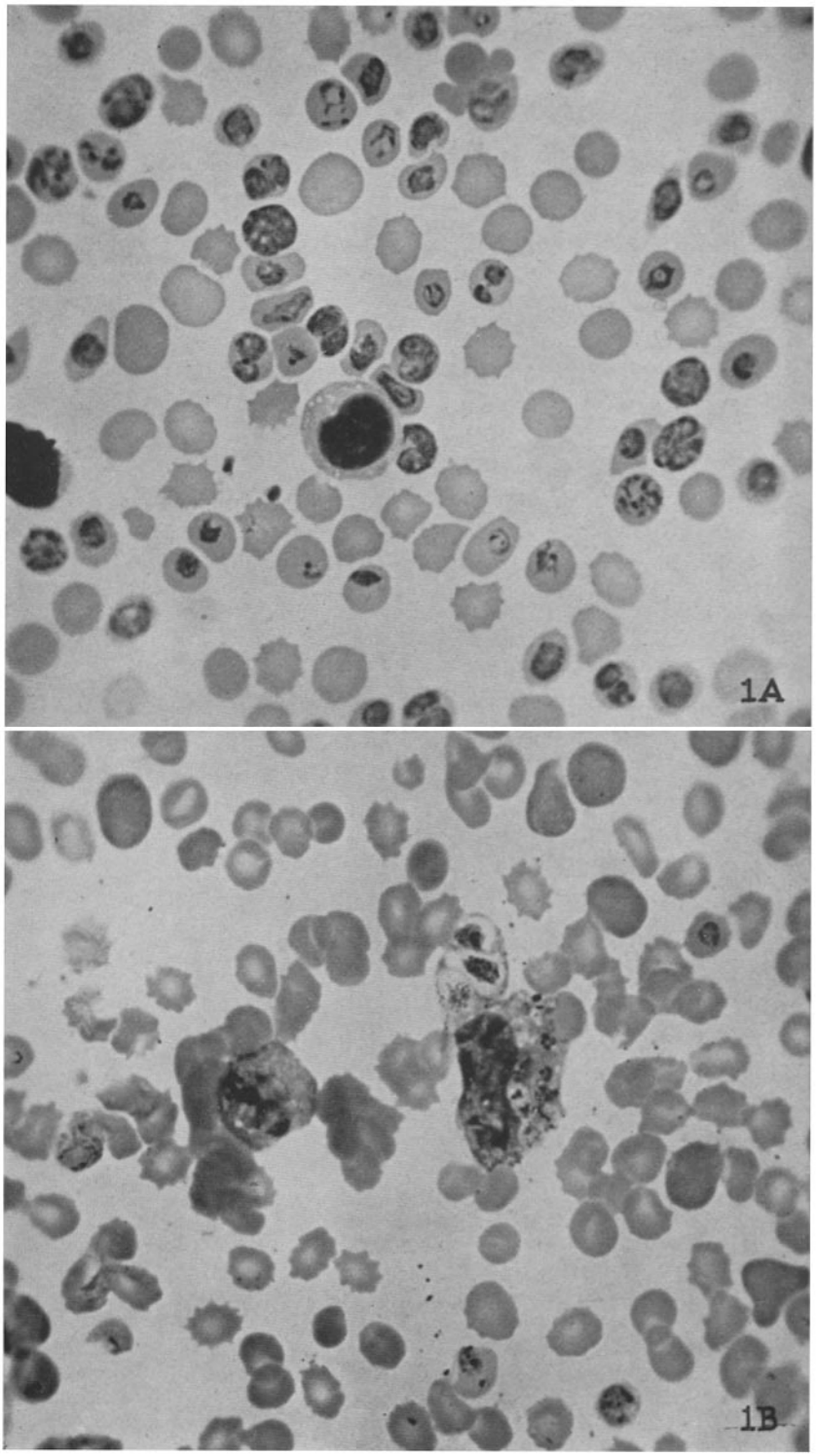
EXPLANATION OF PLATE 1

FIG. 1 A. High percentage of parasitized red blood cells with untreated, mixed *P. knowlesi* and *P. inui* infections.

FIG. 1 B. Macrophages with phagocyted infected red cells in blood from same monkey 48 hours after sulfanilamide therapy.



Photographed by Joseph B. Haulenbeek

(Coggeshall: Selective action of sulfanilamide in malaria)