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Prevention of drug resistance in rodent malaria by the use of drug mixtures

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Development of resistance to chloroquine in rodent malaria is inhibited by giving this compound together with a potentiating mixture of pyrimethamine and sulfadoxine but this does not prevent the development of resistance to the last two compounds. The use of drug mixtures should be explored as a means of "protecting" chloroquine or new blood schizontocides intended for mass chemotherapy against human malaria. However, no general rule can be laid down without testing specific drug mixtures in long-term experiments in a suitable model such as rodent malaria.

The danger of selecting drug-resistant organisms by administering potent antimicrobial agents alone has led to the now accepted practice of using several compounds together in the treatment of bacterial disease. Thus, the accepted procedure for the treatment of tuberculosis has long been to give, for example, a mixture of streptomycin, PAS, and isoniazid. Once resistance has developed it is sometimes possible to overcome this by the judicious use of mixtures of drugs each of which stimulates the action of the others. Bacterial infections that are resistant to sulfonamides, for example, often respond to a mixture of a sulfonamide with a folate reductase inhibitor, such as the combination of trimethoprim with sulfamethoxazole. It has long been known that this type of potentiation is also present when sulfonamides in combination with a suitable folate reductase inhibitor, such as pyrimethamine, are given to patients with acute malaria. However, the role that such mixtures may play in *preventing* the parasites becoming resistant to the individual components of the mixture has been little studied. It is evident that this information must be obtained before a rational decision can be made as to whether this type of mixture should be used for mass drug administration in the control of malaria. The following experiment was designed to compare the rates at which intraerythrocytic asexual stages of the rodent malaria parasite, *Plasmodium berghei*, become resistant to pyrimethamine, sulfadoxine, and chloroquine, given in various combinations.

MATERIALS AND METHODS

A detailed account of the technique used in this experiment has been published elsewhere (1). The principle employed is to expose parasites in albino mice to increasing doses of the drugs, singly or in predetermined mixtures, passaging the parasites into clean mice once weekly. The drugs used in this experiment were pyrimethamine base and sulfadoxine base as a 1 : 3 mixture (PS mixture), and chloroquine phosphate (C). The dose of the drugs is increased in successive passages as long as the subsequent infection is sufficiently high for a further passage to be made. Two series of lines were developed, one starting from the NK65 strain of *P. berghei* (drug-sensitive) and the other from the slightly chloroquine-resistant NS line (2). The following lines were produced:

NK65 series (from *P. berghei* NK65)

NK65 PS exposed to pyrimethamine and sulfadoxine (1 : 3)

NK65 PSC exposed to a fixed combination of pyrimethamine with sulfadoxine (1 : 3), and chloroquine

NK65 C exposed to chloroquine

NS series (from *P. berghei* NS)

NS PS, NS PSC, and NS C exposed as above.

After various intervals, "4-day suppressive tests" (3), were carried out on all lines to determine the effective doses of each drug to each line, and the corresponding levels of drug resistance.

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RESULTS

After 52 weekly passages the following levels of antimalarials were tolerated by parasites of the various NK65 lines in passage, the figures shown being the dose given daily for 6 days of each week:

NK65 PS pyrimethamine with sulfadoxine (1 : 3 mixture), up to 20 mg/kg body weight

PSC pyrimethamine with sulfadoxine (1 : 3), up to 9 mg/kg + chloroquine phosphate, 4 mg/kg

C chloroquine phosphate (since the 12th passage), 60 mg/kg

The NS lines after 45 passages tolerated the following doses:

NS PS pyrimethamine with sulfadoxine (1 : 3), up to 25 mg/kg

PSC pyrimethamine with sulfadoxine (1 : 3), up to 4.5 mg/kg + chloroquine phosphate, 3 mg/kg

C chloroquine phosphate (since the 7th passage), 60 mg/kg

The rate at which the dose could be increased in successive passages is illustrated and summarized in Fig. 1.

At the end of 52 passages the drug responses of the NK65 series in the " 4-day suppressive test " were compared with those of the 45th passage of the NS series. The data obtained are summarized in Table 1.

A subsequent check of the response to pyrimethamine and to the 1 : 3 pyrimethamine/sulfadoxine mixture showed that, by the 63rd passage, the ID_{90} values of the NK65 series had increased markedly. The values were as follows:

	NK65 PS	*NK65 PSC*
pyrimethamine	282	1000
PS mixture	242	400

In order to follow the extent of the changes a final check was made with the " 4-day suppressive test " when the NK65 series had been passaged 67 times and the NS series 57 times. The results are summarized in Table 2. At the time of examination of this material, the NK65 PS line passage material was able to tolerate the 1 : 3 pyrimethamine/sulfadoxine mixture in daily doses of 25 mg/kg and the PSC line daily doses of 8 mg/kg of the mixture with 3 mg/kg chloroquine phosphate. The NS PS line could tolerate 35 mg/kg of the mixture, and the PSC line 8 mg/kg mixture with 4 mg/kg chloroquine.

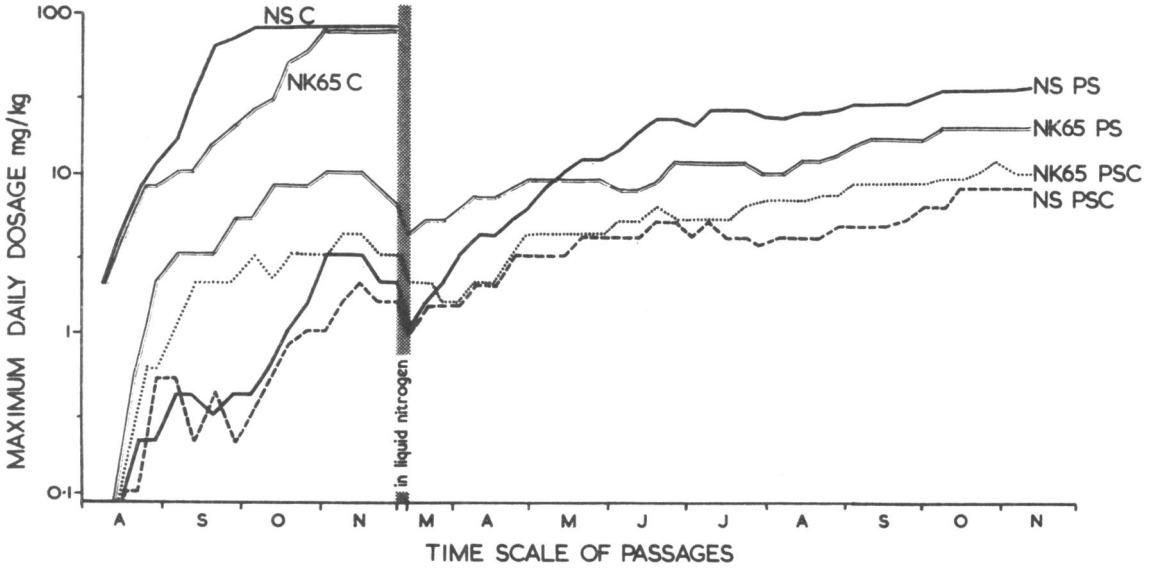

Fig. 1. Increasing exposure to antimalarial drugs in consecutive passages of *P. berghei* NS and NK65 strains. C = treatment with chloroquine alone; PS = treatment with a 1:3 pyrimethamine and sulfadoxine mixture; PSC = exposure to a 1:3 pyrimethamine and sulfadoxine mixture plus increasing dosage of chloroquine. In NK65 and NS PSC lines the maximum dosage of chloroquine reached was 4 mg/kg per day.

Table 1. A comparison of the responses to chloroquine, pyrimethamine, and sulfadoxine in the NK65 and NS series of *P. berghei* strains in the " 4-day suppressive test "

Test drug	52nd passage				45th passage			
	ED_{90} [a]	Resistance I_{90} [b]			ED_{90} [a]	Resistance I_{90} [b]		
	NK65	NK65 PS	NK65 PSC	NK65 C	NS	NS PS	NS PSC	NS C
chloroquine	3.1	0.4	1.0	8.7	40	0.2	0.3	$\geqslant 2$ ($> LD_{100}$)
pyrimethamine	0.4	68	600	1.0	0.12	60	9.2	1.7
sulfadoxine	0.5	29	18	0.3	0.22	118	22	0.9

[a] mg/kg daily × 4

[b] $I_{90} = \frac{\text{90 \% effective dose for resistant line}}{\text{90 \% effective dose for parent strain}}$

(Note: NS parent line is maintained under chloroquine selection pressure)

Table 2. Responses to pyrimethamine and sulfadoxine in the 67th passage of the NK65 series and 57th passage of the NS series of *P. berghei* strains in the " 4-day suppressive test "

Test drug	67th passage			57th passage		
	ED_{90}	Resistance I_{90}		ED_{90}	Resistance I_{90}	
	NK65	NK65 PS	NK65 PSC	NS	NS PS	NS PSC
pyrimethamine	2.6	104	> 280	0.1	210	105
sulfadoxine	0.13	460	230	0.04	> 2500	1250

DISCUSSION

These data may be interpreted as follows. Firstly, it is clear that resistance to chloroquine when used alone develops very rapidly in the NS line but more slowly in the NK65 strain of *P. berghei*. Nevertheless, in both, a high level of resistance is attained rapidly. Resistance to pyrimethamine and to a sulfonamide, when used alone, also develops rapidly. For comparison, this is illustrated in Fig. 2, which summarizes data from earlier work (1, 4). The rate at which resistance develops to a combination of pyrimethamine when given together with chloroquine differs little from that at which resistance develops to pyrimethamine alone (P and CP in Fig. 2). In contrast, chloroquine slows the development of resistance to sulfonamide when the two are given together (OO and CO in Fig. 2), but resistance to the latter still reaches a high level. Pyrimethamine and sulfonamides only act additively with chloroquine. However, pyrimethamine and sulfonamides potentiate each other's action.

It was hoped that the exposure of *P. berghei* to the triple mixture of pyrimethamine, sulfadoxine, and chloroquine would slow down the rate at which the parasites became resistant to these three compounds. When we examine Fig. 1 and the data showing the daily doses of these compounds that could be tolerated by the various lines, it is apparent that this is the case, especially for chloroquine. However, while tolerance to the potentiating mixture of pyrimethamine with sulfadoxine does increase, it does so more slowly than would resistance to the individual components if given alone. Reference to the data in Table 1 and Table 2 reveals a marked difference between the changes in response to pyrimethamine between the NK65 and the NS lines. Contrary to expectation, resistance developed to a much higher level in the NK65 PSC than in the NK65 PS line, whereas the reverse situation applied to the NS PSC and NS PS lines. In the latter case resistance developed to a lower level in the NS PSC line exposed to the triple drug mixture. No such discrepancy is evident when we examine the data for sulfadoxine

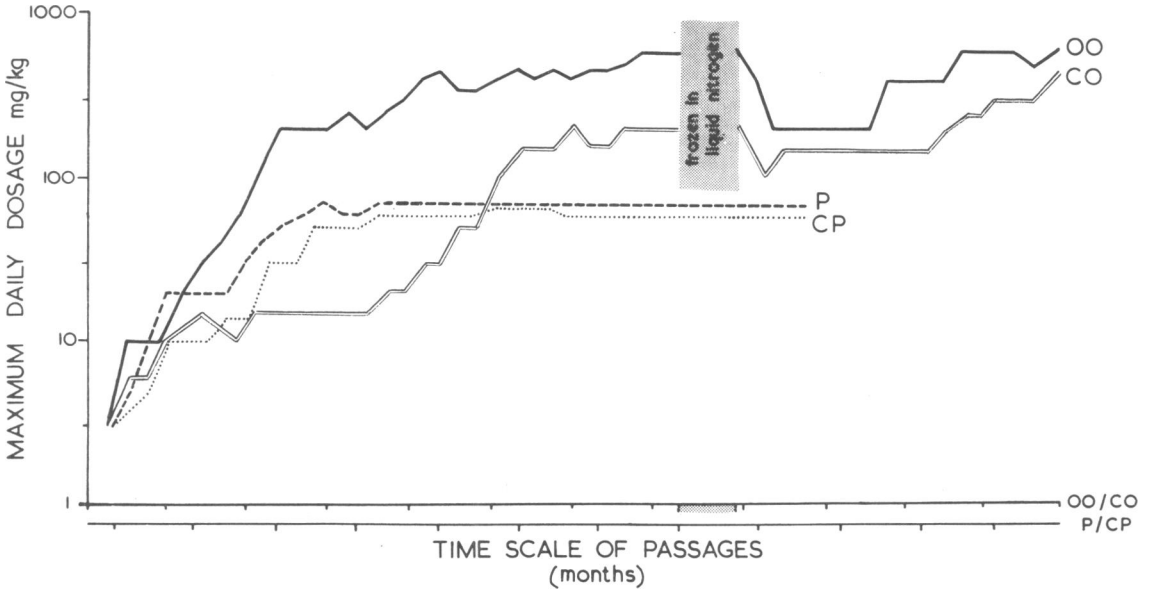

Fig. 2. Increasing exposure to antimalarial drugs in consecutive passages of *P. berghei* NK65.
00 = exposure to sulfaphenazole alone; CO = exposure to chloroquine plus sulfaphenazole (maximum dose of chloroquine reached was 5 mg/kg per day); P = exposure to pyrimethamine alone; CP = exposure to chloroquine plus pyrimethamine (maximum dose of chloroquine reached was 8 mg/kg per day).

in Table 1, where the anticipated result was obtained, the triple mixture protecting the sulfonamide to some degree. However, continuing passage under drug pressure permitted a build-up of resistance to the sulfonamide to a high level in all four strains (Table 2), especially in those of the NS series. The relative resistance of PS and PSC lines was retained, but the overall level of resistance of the NS lines now exceeded that of the NK65 lines. (Some variation is observed in the ED_{90} levels of the parent lines between Table 1 and Table 2. This is probably accounted for by unavoidable variations in the *p*-aminobenzoic acid and folic acid contents of the " standard " diet on which the test mice were fed on the two different occasions.)

Outstanding in these observations is the manner in which *P. berghei* has retained its sensitivity to chloroquine in even the most recent passages of the NK65 PS, NK65 PSC, NS PS, and NS PSC lines. *In the last two cases the parasites have actually regained some degree of sensitivity to chloroquine* as compared with the parent NS line. It will be noted, too, that the NS line in both its 45th and 57th passages is more sensitive than the NK65 line to pyrimethamine and to sulfadoxine. Moreover, the lines that were exposed to chloroquine alone and became highly resistant to this compound (i.e., NK65 C and NS C) retained their sensitivity to both pyrimethamine and sulfadoxine.

On the basis of the experiments described here, it cannot be claimed that the long-term use of a mixture of pyrimethamine, sulfadoxine, and chloroquine (PSC) does more to prevent *P. berghei* becoming resistant to the first two components than does the long-term use of a simple mixture of pyrimethamine with sulfadoxine, although chloroquine *is* " protected " from the development of resistance by the PSC mixture. In neither case is the development of resistance to pyrimethamine or sulfadoxine prevented, although the triple mixture does seem to be somewhat better in this respect, with the notable exception of the NK65 PSC line (noted above). We have shown previously (see Fig. 2) that the simple mixture of pyrimethamine with chloroquine also " protects " chloroquine to some degree (1), but that the mixture of chloroquine with a sulfonamide provides some measure of protection to *both* components (4). Clearly no general rule can be laid down for the use of drug mixtures. Before recommending the administration of mixtures to " pro-

tect" promising new antimalarials, such as the quinolinemethanols and phenanthrenemethanols that are currently undergoing clinical trial, it would be advisable to carry out further long-term experiments of this nature to see what happens, at least in a rodent malaria model, over some 50 or more passages. It will also be necessary, of course, to investigate carefully whether new mixtures alter the levels of tolerability or toxicity of the various components to the host.

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RÉSUMÉ

PRÉVENTION DE LA PHARMACORÉSISTANCE DANS LE PALUDISME DES RONGEURS PAR L'EMPLOI D'ASSOCIATIONS MÉDICAMENTEUSES

La présente recherche a été menée afin de comparer dans quelle mesure les stades asexués intraérythrocytaires du parasite du paludisme des rongeurs, *Plasmodium berghei*, acquièrent une résistance à la pyriméthamine, à la sulfadoxine et à la chloroquine administrées en combinaisons variables. L'information obtenue serait utile en vue de décider de l'emploi éventuel d'associations médicamenteuses de ce genre dans les campagnes antipaludiques de masse.

L'apparition de la résistance à la chloroquine dans le paludisme des rongeurs est inhibée si l'on administre ce médicament en association avec de la pyriméthamine et de la sulfadoxine, mais la pharmacorésistance à l'égard de ces deux derniers composés continue à se développer. Il conviendrait d'étudier la valeur des associations médicamenteuses en tant que moyen de « protéger » la chloroquine ou de nouveaux schizonticides utilisables dans la chimiothérapie de masse contre le paludisme humain. Aucune règle générale ne pourra être définie à cet égard tant qu'on n'aura pas essayé diverses combinaisons de médicaments au cours de recherches de longue durée sur un modèle expérimental comme le paludisme des rongeurs.

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