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Antagonism of serum of mice infected with chloroquine-resistant 'NS' line to the antimalarial action of chloroquine

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Abstract Chloroquine (CQ) solution was separately mixed with the serum of mice infected with chloroquine-resistant 'NS' line (SMNS), the serum of mice infected with chloroquine-sensitive *Plasmodium berghei* ANKA strain (SMCS), and the serum of normal mice (SM). These mixtures were then used in treating mice inoculated with *P. berghei* ANKA strain. The results obtained on d 5 after drug-serum administration showed that the erythrocyte infection rates in the SMNS + CQ, SMCS + CQ, and SM + CQ groups were 32, 16, and 14% respectively. There were significant differences with respect to parasitemia between the SMNS + CQ group and the SMCS + CQ or SM + CQ group ($P<0.05$), suggesting that SMNS may be antagonistic to the antimalarial action of chloroquine. Further study showed that when the 'NS' line lost resistance to chloroquine, the antagonism of SMNS to chloroquine disappeared. The antagonism rates of SMNS to chloroquine, piperaquine, hydroxypiperaquine and pyronaridine were 75, 56, -5, and -17% respectively, a trend similar to that of the results from cross-resistance tests on chloroquine-resistant *P. berghei* ANKA strain. The results indicate that drug-resistant malaria parasites may produce and release a certain 'specific anti-drug substance'.

Key words microbial drug resistance; malaria; *Plasmodium berghei*; drug therapy; chloroquine

Of the many hypotheses proposed for the drug-resistance mechanism of malaria parasites, none has yet been fully confirmed[1]. In the studies of drug resistance of rodent malaria parasites[2,3], we made a comparison of some features of drug-resistant malaria parasites and those of acquired immunity in human beings, and arrived at a new hypothesis: drug-resistant malaria parasites, like lymphocytes producing a specific antibody, may release a certain 'active anti-drug substance' which binds specifically to antimalarials and therefore prevent the drug from entering the body of the parasites. It may be in this way that malaria parasites develop drug resistance[4]. In this paper, experimental evidence supporting our hypothesis is presented.

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Materials and methods

Malaria parasites Chloroquine-sensitive *Plasmodium berghei* ANKA strain and chloroquine-resistant 'NS' line derived from *P. berghei* K 173 strain were obtained from the Department of Medical Protozoology of the London School of Hygiene and Tropical Medicine.

Mice Male mice of Kunming strain weighing 18.3±SD 1.9 g were used and kept at 21℃.

Antimalarials Chloroquine phosphate and piperaquine phosphate were purchased from the Shanghai 14th Pharmacy Factory. Hydroxypiperaquine phosphate was synthesized by the Division of Medicinal Chemistry of our laboratory. Pyronaridine phosphate was kindly provided by the Institute of Parasitic Diseases of the Chinese Academy of Preventive Medicine. Dosages of antimalarials were counted in terms of bases.

Preparation of sera Mice were divided into 3 groups of 30-50 each. The 1st group was inoculated ip with chloroquine-resistant 'NS' line whose resistance level remained 20-30 fold after 3 drug-free passages; the 2nd group received an inoculation of chloroquine-sensitive *P. berghei* ANKA

strain; and the 3rd group which received no inoculation was used as the normal control. The mean erythrocyte infection rates in the 1st and 2nd groups were estimated at 13 and 22%, respectively, just before isolation of sera on d 5 after inoculation. Blood was collected by heart puncture in sterile bottles and kept at 37℃ until fully coagulated. Sera from the coaguated blood were centrifuged at 1500–2000 × *g* for 5 min before their preservation at 4℃.

Usage of drug–sera Antimalarials were dissolved in distilled water and sterilized by filtration. Mixtures of the drug solution with sera were bathed in water at 37℃ for 30 min. The mixtures were then given sc to each mouse which had been inoculated with 2×10^7 mouse red cells parasitized by *P. berghei* ANKA strain 3 h before.

Examination of parasitemia Following drug–serum administration, Giemsa–stained thin films of mouse tail blood were examined daily for parasitemia. The day when drug–serum administration was made was called d 0; following it were d 1, d 2 ···. The erythrocyte infection rate (%) was calculated after examining 1×10^3 to 1×10^4 the mouse red cells.

Results

Effects of different sera on the antimalarial action of chloroquine The first half of Tab 1 showed that the erythrocyte infection rates on 3 consecutive days were 0.6, 4, and 14%, respectively in the SM + CQ group, 1.3, 9, and 32% in the SMNS + CQ group, and 0.7, 4, and 16% in the SMCS + CQ group. There were significant differences in regard with parasitemia on d 4 and d 5 between the SMNS + CQ group and the SM + CQ or SMCS + CQ group ($P<0.05$). As for parasitemia on d 3, significant difference was found only between the SMNS + CQ group and the SM + CQ group ($P<0.05$).

The results exhibited that the antimalarial action of chloroquine in the SMNS + CQ group was reduced, suggesting that a certain 'substance' or 'factor' antagonistic to chloroquine existed in SMNS.

Tab 1. Effects of different mouse sera on the antimalarial action of sc chloroquine (CQ, 5 mg/kg). $\bar{x}\pm SD$. $^{**}P<0.05$ *vs* the SMCS + CQ or SM + CQ group. $^{\dagger\dagger}P<0.05$ *vs* the SM + CQ group. $^{\dagger}P>0.05$ *vs* the SM + CQ group. Male mice of Kunming strain used.

Group	n	Erythrocyte infection rate(%) d 3	d 4	d 5
SM + CQ	9	0.6±0.6	4±4	14±10
SMNS + CQ	10	1.3±0.8††	9±5**	32±17**
SMCS + CQ	10	0.7±0.8†	4±4†	16±13†
SM + CQ	10	1.1±1.0	6±4	19±13
SMns + CQ	10	1.3±1.2†	7±6†	21±9†
SMCS + CQ	10	1.4±1.5†	6±6†	20±13†

The ratio of CQ solution to serum was 1:1 in terms of volume. SM: serum of normal mice; SMNS: serum of mice infected with chloroquine–resistant 'NS' line; SMCS: serum of mice infected with chloroquine–sensitive *P. berghei* ANKA strain; SMns: serum of mice infected with resistance–free 'NS' line.

After the chloroquine–resistant 'NS' line was passaged 6 times without drug pressure, its resistance was almost lost. The line was then used to inoculate mice from which serum (SMns) was subsequently isolated. The experiment was conducted according to the procedures described previously. The second half of Tab 1 summarized the results. No significant differences in parasitemia among the 3 groups (the SMns + CQ, SMNS + CQ, and SM + CQ) were observed, indicating that the antagonism of SMNS to chloroquine was associated with drug–resistant parasites. It is, therefore, reasonable to speculate that chloroquine–resistant 'NS' parasites may produce and release a certain 'substance' which is antagonistic to chloroquine.

Effects of SMNS and SMCS on the antimalarial actions of 4 antimalarials In

order to determine whether the antagonism of SMNS to antimalarials is selective or specific, we conducted an experiment in which SMNS and SMCS were mixed with chloroquine, piperaquine, hydroxypiperaquine, and pyronaridine, respectively. Tab 2 presents the results.

Tab 2. Effects of SMNS and SMCS on the antimalarial actions of sc CQ, piperaquine (PQ), hydroxypiperaquine (HPQ), and pyronaridine (PND). $\bar{x}\pm SD$. *$P>0.05$ *vs* the SMCS + drug group. Antagonism rate (%) = $(P_2-P_1)/P_1\times 100\%$. Male mice of Kunming strain used.

Group	n	Erythrocyte infection rate on d 5(%)	Antagonism rate(%)
SMCS + CQ(5)	9	8±9	75
SMNS + CQ(5)	10	14±13*	
SMCS + PQ(3)	10	16±11	56
SMNS + PQ(3)	10	25±16*	
SMCS + HPQ(3)	8	20±11	−5
SMNS + HPQ(3)	8	19±14*	
SMCS + PND(1)	9	6±5	−17
SMNS + PND(1)	10	5±4*	

P_1 = erythrocyte infection rate (EIR) in the group of SMCS + drug. P_2 = EIR in the group of SMNS + drug. The ratio of drug solution to serum was 1.7:1 in terms of volume. Drug dosage (mg/kg) in parentheses.

The antagonism rates of SMNS to chloroquine, piperaquine, hydroxypiperaquine, and pyronaridine were 75, 56, −5 and −17%, respectively. The results suggest that the antagonism of SMNS to antimalarials is specific.

Discussion

The antagonism of serum of mice infected with chloroquine-resistant 'NS' line to the antimalarial action of chloroquine indicates that chloroquine-resistant malaria parasites probably produce a certain 'substance' which is antagonistic to chloroquine. The fact was well demonstrated by the results showed in the first half of Tab 1. Further study suggests that the antagonism of SMNS to antimalarials was not aimless but selective or specific. Although no significant differences in parasitemia between the groups as shown in Tab 2 was found, the antagonism-rate trend similar to that of cross-resistance tests on chloroquine-resistant *P. berghei* ANKA line[5] shows that the data in Tab 2 are meaningful. Besides, the reason of no significant differences seems to be associated with the quantity of serum in drug-serum mixtures. In the experiment of Tab 1, the ratio of drug solution to serum was 1:1, while in the experiment of Tab 2, the proportion was 1.7:1 in terms of volume.

The recently accepted 'FP-hypothesis' explains chloroquine resistance in *P. berghei* by a lack of ferriprotoporphyrin IX (FP) production[6]. But the results obtained from our experiments did not support the hypothesis. According to the hypothesis, FP is only produced in pigmented malaria parasites and once it binds chloroquine, the toxic FP-chloroquine complex which impairs the ability of parasites is formed. If the theory is true, the expected results from our experiments would be much different from those presented in this paper, that is, parasitemia in the SM + CQ group would be the highest among the 3 groups because of the lack of FP in SM, and parasitemia in the SMCS + CQ group would be the lowest among the 3 groups because SMCS may contain a larger amount of FP than SMNS.

It is concluded that chloroquine-resistant malaria parasites may produce and release certain 'specific anti-drug substance'. It therefore provides another way to explain the drug-resistance mechanism of chloroquine-resistant malaria parasites. Further work should be done on the other drug-resistant strains, including both animal and human malaria parasites, especially *Plasmodium falciparum*.

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感染抗氯喹 'NS' 株小鼠血清对氯喹抗疟药效的拮抗作用

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提要　分别用感染抗氯喹 'NS' 株小鼠血清(SMNS)、感染氯喹敏感株小鼠血清(SMCS)和无原虫感染的正常小鼠血清(SM)与氯喹(CQ)水溶液的混合物治疗感染正常伯氏疟原虫小鼠。给药后 d 5 涂片检查，SMNS + CQ 组，SMCS + CQ 组和 SM + CQ 组原虫感染率分别为：32，16 和 14%。SMNS + CQ 组与其它两组原虫感染率比较，相差显著($P<0.05$)，提示 SMNS 对氯喹药效具有拮抗作用。进而发现当 'NS' 株对氯喹抗药性消失后，SMNS 对氯喹药效的拮抗作用亦消失。另外，还同时测试了 SMNS 对氯喹、喹哌、羟基喹哌和咯萘啶的拮抗率，依次为 75、56、－5 和－17%，此结果与抗氯喹伯氏疟原虫 ANKA 株对这些药物的交叉抗性程度的趋势相似。本文结果提示，抗药性疟原虫可能产生某种"特异性抗药物质"。

关键词　微生物抗药性；疟疾；伯氏疟原虫；药物治疗；氯喹