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In Vivo Activity of Ajoene against Rodent Malaria

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Ajoene (4,5,9-trithiadodeca-1,6,11-triene 9-oxide), a product initially isolated from extracts of garlic (*Allium sativum*), was tested for its antimalarial activity in vivo in a well-characterized murine model. A single ajoene dose of 50 mg/kg, on the day of infection, suppressed the development of parasitemia; there were no obvious acute toxic effects from the tested dose. The combination of ajoene (50 mg/kg) and chloroquine (4.5 mg/kg), given as a single dose on the day of the infection, completely prevented the subsequent development of parasitemia in treated mice.

Malaria parasites resistant to various drugs are widespread in South America, Asia, and Africa (15). In particular, the chloroquine (CQ)-resistant strains of *Plasmodium falciparum* have become a major public health problem around the world (11, 15). Hence, the search for new antimalarial therapies is a high-priority task for the control of the disease. Ajoene (4,5,9-trithiadodeca-1,6,11-triene 9-oxide), an organosulfur compound derived from garlic (*Allium sativum*) (1, 7), is a well-known inhibitor of platelet activation (2–4) and also has significant antifungal (10, 12, 16), antitrypanosomal (14), and antiviral (13) activities.

We investigated the potential anti-*Plasmodium berghei* activity of ajoene in vivo. The results show that when used alone, ajoene displays a moderate anti-*P. berghei* activity. However, when used in combination with a noneffective dose of CQ, ajoene synergistically enhances the susceptibility of the parasite to this drug.

MATERIALS AND METHODS

Ajoene. Synthetic ajoene (M_r, 234) was prepared as previously described (1, 1a, 3), dissolved in ethanol, and diluted eightfold with Intralipid (200 g of fractionated soybean, 12 g of fractionated egg phospholipids, 22 g of glycerol USP, 1,000 ml of water q.s.p) (Kavi Vitrum, Stockholm, Sweden). The final concentration of ethanol was less than 1% (vol/vol).

Animals, parasites, and infection. Female BALB/c mice 8 to 10 weeks old were obtained from the animal breeding unit of the Instituto Venezolano de Investigaciones Científicas. Mice were kept in plastic cages and received standard food and water ad libitum. The strain of *P. berghei* used was originally obtained from N. H. Swellengrebel, Institute of Tropical Medicine "Prince Leopold," Belgium, in 1950 and since then has been maintained in the Venezuelan Institute of Tropical Medicine "Felix Pifano" by passage in outbred albino mice. It was given to the Instituto Venezolano de Investigaciones Científicas in 1988. In our laboratory parasites are kept frozen in liquid nitrogen as a 1:2 dilution of infected blood (10 to 15% infection) in 28% glycerol–3% sorbitol. The parasite was passaged once or twice in BALB/c mice before use in each experiment. Mice received 10^5 parasitized cells injected intraperitoneally. The parasitemia was monitored regularly by counting numbers of infected erythrocytes per 1,000 erythrocytes on tail blood smears stained with Giemsa stain. Results are expressed as the percentage of infected cells, or inhibition of parasitemia, calculated from the following equation: percent inhibition = 100 − {[estimated number of infective parasites treated with compounds/estimated number of infective parasites treated with no compound (control)] × 100} (6).

Animal experiments. All experimental mice were infected at random before being divided into groups of 7 to 10. Mice were treated by intraperitoneal injection of ajoene given on the day of inoculation (day 0). The time between the inoculation of parasites and drug injection was 60 min. The effectiveness of CQ alone at a dose of 4.5 mg/kg (8) or in combination with various concentrations of ajoene was studied by using the same experimental protocol described for ajoene. CQ diphosphate (CQ; Sigma Chemical Co.) was diluted in 0.9% (wt/vol) NaCl to give the dose required in 0.1 ml for 10 g of mouse and was administered intraperitoneally. Experiments were repeated at least three times. Data from a representative experiment are given.

RESULTS

Drug toxicity. On the basis of the results of a set of preliminary experiments, ajoene doses of up to 50 mg/kg were used. No signs of toxicity were observed in 30 days.

Inhibition of parasite growth by ajoene. In BALB/c mice infected with 10^5 parasitized erythrocytes, systemic parasitemia became apparent (1%) on day 2 and increased to 24 ± 2.8% infected cells on day 6 after the inoculation. Ajoene (50 mg/kg) given at the start of the infection (day 0) had a marked schizonticidal activity against *P. berghei*. Thus, the parasite growth was considerably suppressed from days 2 to 12, with the level of parasitemia in ajoene-treated mice on day 12 after inoculation being significantly lower ($P < 0.001$) than that in controls as determined by Student's t test. Infected mice that received only the vehicle showed a course of parasitemia similar to that of uninfected controls (data not shown). Comparison of the slopes of the regression lines calculated for the courses of parasitemia of control (slope = 4; $r = 0.97$; $P = 0.001$) and ajoene-treated (slope = 1.3; $r = 0.93$; $P = 0.005$) mice supported the existence of an inhibitory effect of ajoene on parasite growth (Fig. 1). In the next experiments the effect of various doses (6.25 to 50 mg/kg) of ajoene on parasite growth was examined. Mice inoculated with 10^5 parasitized cells were given a single dose of ajoene, and their parasitemia was determined on day 6 after inoculation. The results showed that the inhibition of parasite growth by ajoene is dose dependent. Maximal inhibition of parasitemia was observed in

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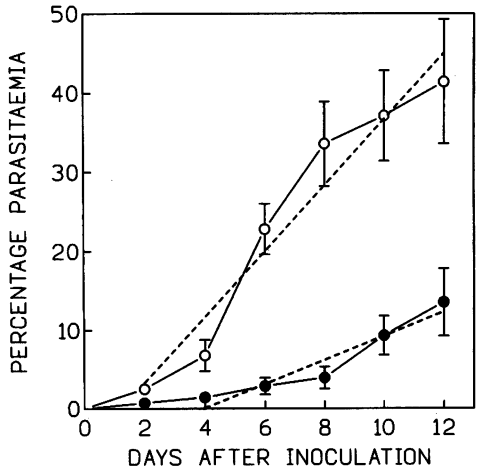

FIG. 1. Effect of ajoene on *P. berghei* parasitemia. BALB/c mice were infected with 10^5 parasitized erythrocytes and given a single dose of ajoene (50 mg/kg) on the day of inoculation. Parasitemias of control (○) and ajoene-treated (●) mice, up to 12 days after inoculation, are shown. Vertical bars indicate 1 standard deviation from the mean; n = eight mice per group. Regression lines (– –) are given for the course of parasitemia of control (slope = 4.2; r = 0.97; P = 0.001) and ajoene-treated (slope 1.2; r = 0.93; P = 0.0054) mice.

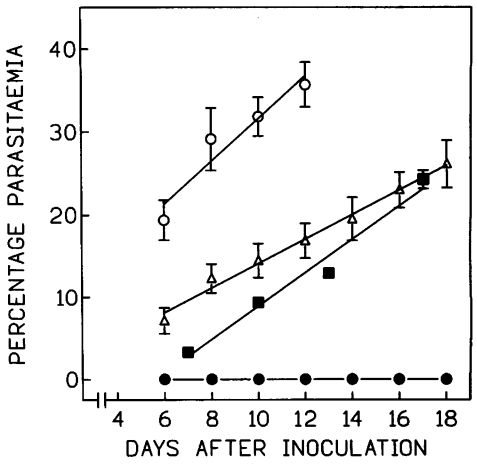

FIG. 3. Effect of ajoene and CQ on *P. berghei* parasitemia. Regression lines are shown for the course of parasitemia of control, untreated mice (○) (slope = 2.6; r = 0.96; P = 0.04) and of infected mice treated on the day of inoculation with a single dose of CQ (4.5 mg/kg) (△) (slope = 1.5; r = 0.99; P < 0.0001), with a single dose of ajoene (50 mg/kg) (■) (slope = 2.03; r = 0.98; P = 0.01), or with a single dose of a combination of ajoene (50 mg/kg) and chloroquine (4.5 mg/kg) (●). All mice were inoculated with 10^5 parasitized erythrocytes. Vertical bars indicate 1 standard deviation from the mean; n = seven mice per group.

the group receiving the highest dose of ajoene (50 mg/kg), but even a relatively low dose of ajoene (6.25 mg/kg) significantly (P < 0.001 by Student's t test) suppressed the development of parasitemia compared with that of controls. Regression analysis indicated an inverse relationship between the level of parasitemia and the increase in the ajoene dose (slope = −0.50; r = −0.95; P < 0.012) (Fig. 2). It was estimated that for each 1-mg/kg increase in ajoene, parasitemia declined by 0.5%.

Suppressive activity of ajoene and CQ against *P. berghei*. Although a single dose of ajoene given on the day of inoculation had a marked anti-*P. berghei* effect, it failed to eliminate the parasite. It was interesting, therefore, to evaluate whether ajoene could enhance the suppressive activity of CQ in vivo. As shown in Fig. 3, a single dose of CQ (4.5 mg/kg) in combination with ajoene (50 mg/kg) given on day 0 completely prevented the development of parasites, leading to 100% survival 60 days after inoculation and no detectable parasitemia. Comparable results with CQ alone were obtained after four doses (4.5 mg/kg), given daily from day 0 to day 3 after inoculation (data not shown). A single dose of CQ (4.5 mg/kg) on day 0 suppressed the growth of parasites, but suppression was very slight compared with that achieved by coadministration of ajoene. All mice died between days 16 and 20 after infection. The slopes of the regression lines calculated for the courses of parasitemia of controls (slope = 2.6; r = 0.96, P = 0.04) and mice treated with either ajoene alone (slope = 2.03; r = 0.98; P = 0.01) or CQ alone (slope = 1.49; r = 0.99; P < 0.0001) indicate that the antimalarial activity of the combination of ajoene and CQ is not a simple additive effect (Fig. 3).

Ajoene at a concentration (12.5 mg/kg) that affected only 29% of parasites (day 6) was sufficient to inhibit the development of parasitemia by 85% when combined with a dose of CQ (2.8 mg/kg) that reduced parasitemia by 21%. No inhibitory effect of CQ was detected on day 8. However, mice given CQ and ajoene exhibited more than 50% inhibition of parasite growth (Fig. 4). Comparisons by one-way analysis of variance of the inhibition of parasitemia found on day 6 showed that the difference among the group means was highly significant (P < 0.0001). Calculation of the Bonferroni P value indicated that the inhibition of parasitemia observed in the group which received a single dose of the combination of 12.5 mg/kg of ajoene plus 2.8 mg/kg of CQ was significantly higher than those observed in the groups treated with the corresponding dose of either CQ alone (P < 0.001) or ajoene alone (P < 0.001). It thus follows that ajoene increased the susceptibility of *P. berghei* to CQ.

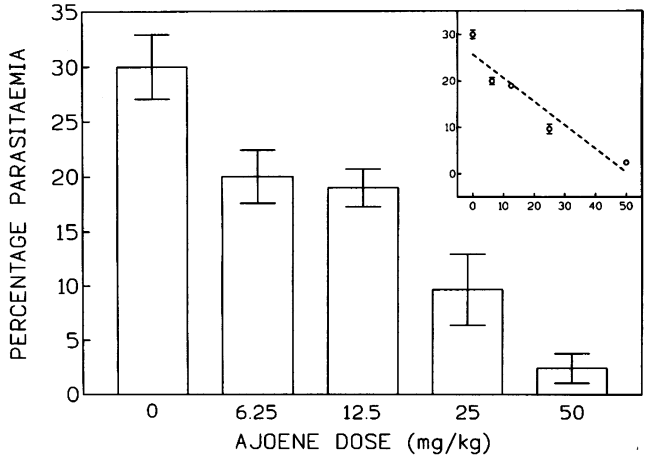

FIG. 2. Effect of various doses (6.25 to 50 mg/kg) of ajoene on inhibition of parasite growth. The average parasitemias of control and experimental mice on day 6 after inoculation with 10^5 parasitized erythrocytes are shown. Ajoene was given as a single dose. Vertical bars indicate 1 standard deviation from the mean; n = 10 mice per group. The inset shows the regression line for the dose-response relationship (slope = −0.50; r = −0.95; P = 0.01).

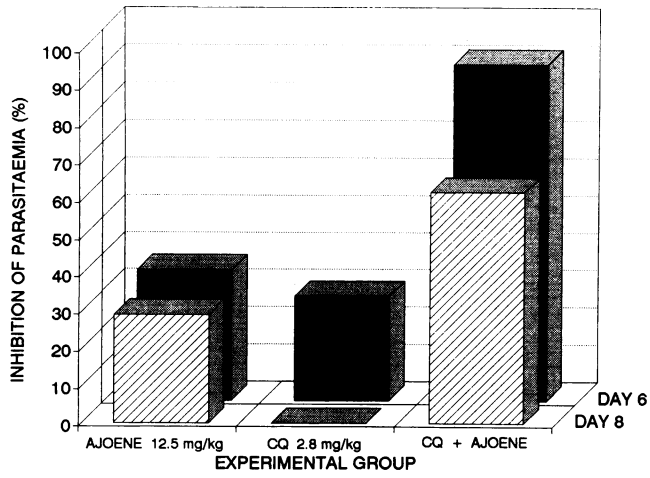

FIG. 4. Synergistic activity of ajoene and CQ against *P. berghei*. Mice were treated with ajoene (12.5 mg/kg) and CQ (2.8 mg/kg) on the day of inoculation. The percentages of inhibition of parasitemia on days 6 and 8 after inoculation are shown. Vertical bars indicate 1 standard deviation from the mean; n = eight mice per group.

DISCUSSION

The results of this study show that ajoene has activity against *P. berghei* in vivo. The patterns of parasitemia of control mice and mice treated with ajoene (50 mg/kg) indicate a distinction between the two groups. Thus, parasitemia rose abruptly, reaching about 30% in untreated controls by day 10 after infection. In contrast, the parasitemia rose gradually in the group given ajoene and by day 10 was below 10%. The experimental data described here do not provide information on the possible mechanism of action of ajoene against *P. berghei*. However, studies on the interaction of ajoene with platelet membranes have shown that this molecule localizes deep in the layer, inducing a disordering effect on the deep part of the membrane (9). Ajoene may alter protein and lipid trafficking in the parasite and host cell membranes (5), leading to irreversible damage of parasites. Of major importance is the fact that the combination of ajoene and a noncurative dose of CQ completely prevented the development of parasitemia. The evidence supports the notion that there is a synergistic effect of ajoene and CQ. One dose of ajoene (50 mg/kg) alone exhibited antimalarial activity but failed to cure the infection. Similarly, one dose of CQ (4.5 mg/kg) was subcurative. However, mice were completely cured of an otherwise fatal infection by coadministration of ajoene (50 mg/kg) with CQ (4.8 mg/kg). The successful antimalarial activity of a subcurative dose of CQ (2.8 mg/kg) with a very low dose of ajoene (12.25 mg/kg) further supports the use of ajoene as a CQ synergist. Nevertheless, the pharmacokinetics of ajoene have not been studied, and further studies are required to determine whether ajoene affects the pharmacokinetics of CQ or vice versa.

In summary, ajoene alone inhibited the development of parasitemia induced in mice by the inoculation of *P. berghei*-infected erythrocytes but failed to cure the infection. However, ajoene substantially improved the antimalarial activity of CQ. The mechanism underlying the synergistic action of CQ and ajoene is not yet clear. Regardless of the actual molecular mechanism(s) involved, these results demonstrate a novel way to potentiate CQ effects which may be of particular interest when dealing with drug-resistant parasites. Our strain of *P. berghei* is CQ susceptible, and further experiments are needed to establish whether ajoene is also able to potentiate the CQ effect in CQ-resistant parasites.

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