ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1997, p. 1778–1782 Vol. 41, No. 8
0066-4804/97/$04.00+0

Effect of Tryptophan-*N*-Formylated Gramicidin on Growth of *Plasmodium berghei* in Mice

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Received 9 July 1996/Returned for modification 20 February 1997/Accepted 8 May 1997

The effect of tryptophan-*N*-formylated gramicidin (NFG) on the growth of *Plasmodium berghei* in mice was tested in three different experiments. NFG was shown to be capable of inhibiting the growth of the parasite in a dose-dependent way, although its action did not result in elimination of the parasite and was only temporary, preventing mice from early death, presumably due to cerebral malaria, but not from fatal generalized malaria. Intriguingly, a similar observation was made with two other drugs, (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine, an inhibitor of viral and eukaryotic DNA polymerases, and the presumed topoisomerase II inhibitor, a bisquaternary quinolinium salt. A rise in the level of parasitemia after 8 days, despite continued treatment, was not due to parasite-induced reticulocytosis, as demonstrated in experiments in which this condition was induced artificially. NFG was added in the form of lipid vesicles in which the peptide had been incorporated. The inhibitory action of NFG was not modulated by the lipid composition of the vesicles. Control experiments did not demonstrate any toxicity of NFG when it was administered in lipid vesicles. The main observation is that NFG is able to inhibit the growth of a malaria parasite in vivo at concentrations that are well tolerated by the host.

Gramicidins are 15-amino-acid-long linear peptides which spontaneously adopt an α-helical configuration when inserted into a hydrophobic environment, such as a lipid bilayer. The subsequent formation of dimers of those α-helical molecules results in the formation of membrane-spanning channels, thus allowing for the passive diffusion of monovalent cations through that membrane. They are being used as antibiotics, but their hemolytic character limits that application (17). The toxicity of gramicidin can be reduced considerably by structural modifications, and the rationale for testing one of those derivatives, tryptophan-*N*-formylated gramicidin (NFG), as an antimalarial agent is as follows.

(i) Formylation of the tryptophan moieties of gramicidin results in a molecule which, upon insertion into the membrane of a normal erythrocyte, hardly modifies the lipid bilayers and consequently has a reduced ionophoretic capacity and considerably reduced hemolytic properties (11).

(ii) The channel lifetime of gramicidin and its derivatives increases when lipid bilayer surface pressures decrease (8, 15).

(iii) The surface pressure in the membrane of malaria parasite-infected erythrocytes is known to be lower than that in uninfected cells (9).

One may therefore expect that NFG will be unable to form stable channels in the tightly packed membrane of uninfected erythrocytes, whereas their introduction into the weakened membranes of infected cells will result in the formation of stable channels and a substantial leak of K^+ ions. This loss could be lethal for the parasite. It has been shown previously that NFG causes growth inhibition of *Plasmodium falciparum* in vitro by inducing potassium efflux from infected erythrocytes (13) and that this growth inhibition can be obtained at drug concentrations which are not lytic and which do not result in an irreversible damage of uninfected cells (11, 13). Preliminary experiments, furthermore, showed an inhibitory effect of the drug on the growth of the malaria parasite in vivo in a mouse model.

The present study describes this latter effect in more detail. Emphasis is given to the mode of administration of the drug. NFG is completely insoluble in water, and as a carrier of the drug we selected a commonly used lipid vesicle preparation consisting of NFG, distearoylphosphatidylcholine (DSPC), cholesterol, and monosialoganglioside (GM_1) (4, 5) or the much less expensive phosphatidic acid (PA) at molar ratios of 0.5, 10, 5, and 1, respectively (1, 12), to study the effects of dose and the regimen of NFG administration on the growth of *Plasmodium berghei* in mice. Since complete eradication of parasites was not obtained, follow-up treatments with NFG were given to maintain parasitemias at low levels so as to give the mice a chance to develop protective immunity. *P. berghei* is known to accumulate preferentially in reticulocytes (2), and because the data suggest that by accumulating in reticulocytes the parasite might escape from the drug, the influence of reticulocytes on drug treatment was examined. To this end, reticulocytosis was induced in a group of mice by treatment with phenylhydrazine prior to infection and drug treatment.

The results of these studies are described and compared with data obtained in parallel experiments with two other drugs, (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA), an inhibitor of viral and eukaryotic DNA polymerases, and a bisquaternary quinolinium salt (BQQ), the presumed topoisomerase II inhibitor (3).

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MATERIALS AND METHODS

Materials. Gramicidin A′, DSPC, egg PA, GM_1, and cholesterol were obtained from Sigma (St. Louis, Mo.). Gramicidin A was purified from gramicidin A′ as described by Vogt et al. (17). ^{14}C-labelled gramicidin was prepared as

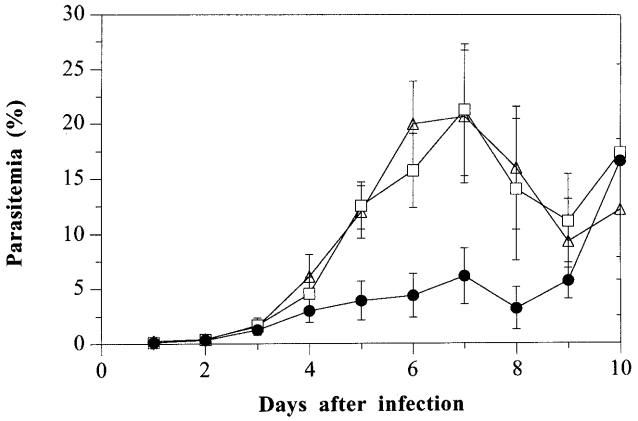

FIG. 1. Effect of four doses of 3 mg of NFG/kg of body weight given i.p. at intervals of 24 h (experiment 1) on the average level of parasitemia in a group of 10 mice (●) compared with the average level of parasitemia in a group of 5 untreated mice (infection control; △) and a group of 5 mice treated with vesicles without NFG (the mice received lipids at amounts equivalent to those received by NFG-treated mice; vesicle control; □). Treatments started 24 h after infection. Means ± SDs are given.

described by Tournois et al. (16). NFG and ^{14}C-labelled NFG were obtained by formylation of the four tryptophans of gramicidin A with formic acid (8). NaCl and phenylhydrazine-HCl (PHZ) were from Merck (Darmstadt, Germany).

Preparation of drug suspensions. NFG-containing lipid vesicles were prepared as follows. In a 20-ml glass tube, a solution containing 30 μmol of DSPC, 2.8 μmol of GM_1 (or PA in experiment 2 only), 15 μmol of cholesterol, and 0.75 μmol of NFG in chloroform-methanol was evaporated to dryness under a nitrogen stream. The dry NFG-lipid film was hydrated with 2 ml of 150 mM NaCl solution (pH 6 to 7, sterile) and was suspended by vortexing and bath sonication (15 to 20 min). This vesicle suspension was sonicated for 30 min with a Branson sonifier with a microtip at 80 W under nitrogen in ice water. Titanium particles originating from the sonicator tip and heavy NFG-lipid particles were removed by centrifugation at 180 × *g* for 5 min. Vesicles without NFG were prepared in the same way. Drug suspensions were stored at 4°C and were used within 5 days.

Analysis of drug suspensions. The concentration of NFG varied among the experiments due to differences in the amount of NFG that was pelleted by centrifugation of the sonicated preparations. [^{14}C]NFG-containing sonicated preparations were made in parallel and under conditions identical to those described above. The amount of radioactivity determined in these preparations was taken to calculate the concentration of the drug in the preparations that contained the unlabelled NFG. Furthermore, the antimalarial activities of both types of preparations were tested in vitro, as described previously (12).

The phospholipid concentration of the NFG-containing vesicle suspensions was 12 to 16 mM, as determined by measuring the phospholipid phosphorus concentration as described by Rouser et al. (14).

The particle size of the NFG-containing vesicles was calculated from dynamic light scattering data, as described elsewhere (11). The average sizes of NFG-containing vesicles varied from 170 to 190 nm, and those of vesicles without NFG varied from 110 to 120 nm.

Animals. Male BALB/c mice (weight, 20 g) were obtained from the common animal laboratory (GDL), Utrecht, The Netherlands. The animals were kept in cages holding 5 to 10 mice each and were fed a standard diet, with water available ad libitum.

Infections. Mice were infected by intraperitoneal (i.p.) injection of 10^6 parasitized erythrocytes from mice infected with the ANKA strain of *P. berghei* (7). None of the infections were synchronized. Every 24 h, thin smears were made from tail blood, stained with Giemsa, and observed by light microscopy (oil immersion; magnification, ×1,000). Parasitemias were estimated from approximately 800 observed erythrocytes. The stage distribution of the parasites was determined by microscopic observation of at least 100 parasites.

NFG treatment. Mice with parasitemias of 0.1 to 0.2% were injected i.p. with 0.2 ml of NFG suspension or of a lipid equivalent of vesicles without NFG (vesicle control). At approximately 24 h postinfection (p.i.) a first dose of 3 to 7.5 mg of NFG/kg of body weight was given. This was repeated several times, as indicated in Results. Infected untreated mice and noninfected identically treated mice served as infection controls and toxicity controls, respectively.

PHZ treatment. In one experiment, two groups of mice received 50 mg (0.1 ml) of PHZ/kg of body weight i.p. 3 days before infection in order to induce reticulocytosis. At day 2 and day 7 p.i., reticulocytes were counted in brilliant cresyl blue-stained thin blood smears and were expressed as a percentage of total erythrocytes.

RESULTS

In a pilot experiment mice were given suspensions of NFG by i.p. or intravenous (i.v.) injection on 4 consecutive days, starting at 24 h p.i. with *P. berghei*. Parasitemias were determined starting on the fifth day and were determined until the mice died. Four doses of 21 nmol of NFG given i.v. (each day a dose of 1.2 mg/kg of body weight) produced 84% inhibition and four doses of 67 nmol of NFG given i.p. (four doses of 3.9 mg/kg of body weight) produced 100% inhibition of parasite growth. Parasites were, however, not completely eradicated, because on day 6 p.i. parasites were observed in all blood smears. Since there was no apparent advantage of i.v. over i.p. administration, the latter was used in all following experiments by using different schedules, but all starting at 24 h p.i.

Experiment 1. A group of 10 infected mice was given four doses of 3 mg of NFG/kg of body weight on 4 consecutive days. Control groups consisted of five mice. The levels of parasitemia were determined daily (Fig. 1). At day 1 p.i. the levels of parasitemia were 0.1 to 0.2% in all groups. In the two infected control groups the levels of parasitemia increased fast to approximately 20% on day 7, dropped again to approximately 10% on day 9, and then rose to a maximum of approximately 44% on day 14, staying that high until, within 9 days thereafter, the mice died. Treatment with NFG, starting at 24 h p.i., inhibited the growth of the parasites for 8 days. However, the levels of parasitemia did not drop to 0%, and from day 9 they ran parallel in all infected groups.

Twice, on days 3 and 5, parasites were differentiated as to their stage of erythrocytic development both in the treated and in the control mice. In the latter the percentage of ring stages of total parasite number varied from 14.7% ± 0.9% on day 3 to 20.8% ± 2.3% on day 5, whereas in the NFG-treated mice this percentage was almost more than doubled (23.5% ± 4.0% and 45.5% ± 15.1%) a difference that was statistically different compared to the values for the control mice (two-sample *t* test;

TABLE 1. Schedules of treatment for groups of six mice given several doses of 7.5 mg of NFG/kg of body weight[a]

Group	Infection	Initial treatment	Follow-up treatment	Mean survival time (days [mean ± SD])
Infection control	+			12 ± 7
GM_1-1×	+	One dose at day 1	One dose at day 9	18 ± 5
GM_1-4×	+	Four doses at days 1 and 2	Four doses at days 10 and 11	17 ± 2
GM_1 toxicity control	−	Four doses at days 1 and 2	Four doses at days 10 and 11	
PA-1×	+	One dose at days 1	One dose at day 7	20 ± 5
PA-4×	+	Four doses at days 1 and 2	Four doses at days 10 and 11	23 ± 5
PA toxicity control	−	Four doses at days 1 and 2	Four doses at days 10 and 11	

[a] GM_1 and PA indicate that mice were given NFG incorporated into vesicles containing GM_1 or PA, respectively. Repeat doses were given at intervals of 10 h.

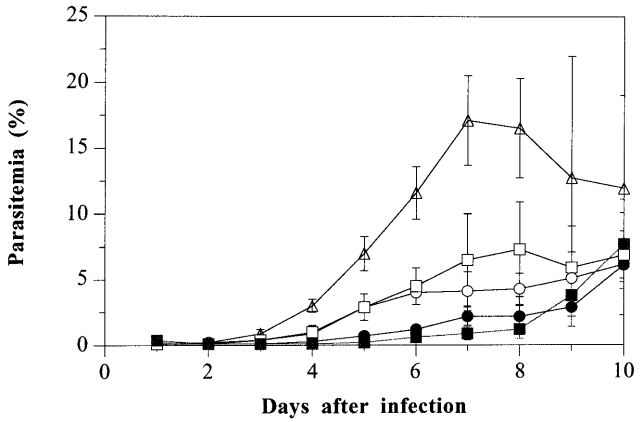

FIG. 2. Effect of a single dose (GM_1 [○] and PA [□]) or of four doses (GM_1 [●] and PA [■]) of 7.5 mg of NFG/kg of body weight given i.p. at intervals of 10 h (experiment 2) on the average level of parasitemia in groups of six mice infected with *P. berghei*. A group of six infected mice left untreated served as a control group (△). NFG incorporated into vesicles containing GM_1 or PA was administered. Means ± SDs are given. The schedules of treatment indicated in Table 1 were followed.

$P < 0.01$), demonstrating that non-ring stages (trophozoites and/or schizonts) are more sensitive than ring stages.

Treatment with vesicles without NFG had no effect on the growth of the parasites. Due to very large standard deviations (SDs) in two of the three groups, there was no significant difference (two-sample *t* test; $P < 0.05$) in the average survival of the mice in the three groups: 14.4 ± 8.0 and 15.2 ± 5.7 days in the infection control and vesicle control groups, respectively, and 21.8 ± 1.6 days in the NFG-treated group. It is relevant, however, that early death occurred only in the nontreated groups. All mice in the toxicity control group looked very healthy throughout the experiment, and all were alive on day 25.

Experiment 2. In order to avoid the situation in which treatment was in phase with the 24-h erythrocytic cycle of the parasite (see Discussion), the interval between successive administrations of NFG was shortened to 10 h. The exact treatment schedules are given in Table 1. Results are given in Table 1 and Fig. 2 and 3.

Again, the levels of parasitemia in the infection control group rose rapidly from day 2 onward and, after a temporary decline on days 8 to 10 as in experiment 1, reached top levels on day 14. Four of the six mice died before day 10 (presumably from cerebral malaria), and the other 2 died on day 22. NFG treatment postponed the day of first appearance of death (day 8 in the infection control group) by about a week (21 of the 22 NFG-treated mice were still alive on day 14) and inhibited the growth of the parasites during the first week of infection in a dose-dependent way, causing in a few mice (that were positive on day 1) disappearance of the parasite from the blood after treatment with the higher dose. However, the parasite was not cleared from the blood of any of the mice, and from day 10 onward the levels of parasitemia rose fast in all groups, despite follow-up treatments as soon as parasitemia levels reached 5%. All treated mice with one exception died between days 15 and 25. Differences in mean survival times between infected groups were statistically not significant (two-sample *t* test; $P < 0.05$) due to large SDs (Table 1). PA instead of GM_1 in the NFG-containing vesicles did not diminish the effect of NFG on the initial growth of the parasites or on survival time; rather, the reverse was true (although the differences were not significant). On day 35, the end of the experiment, all mice in the toxicity control groups were in good condition.

Experiment 3. Reticulocytosis was induced by PHZ injection, as indicated in Materials and Methods. PHZ causes destruction of erythrocytes, thereby stimulating erythroid tissue

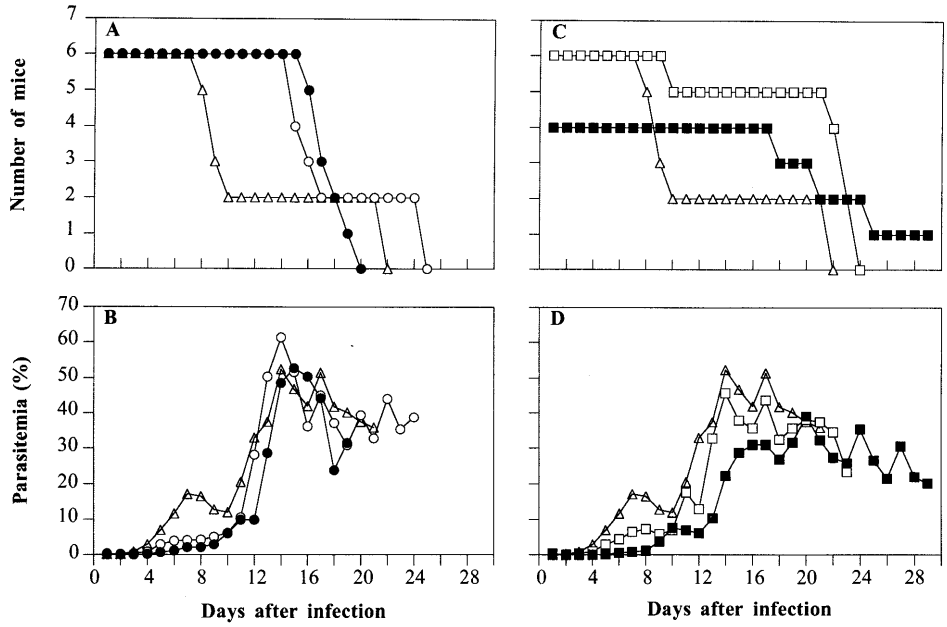

FIG. 3. Survival (A and C) and parasitemia (B and D) for groups of six mice after treatment with several doses of 7.5 mg of NFG/kg of body weight i.p. NFG was incorporated into vesicles containing GM_1 (A and B) or PA (C and D). Schedules of treatments are given in Table 1. △, infection controls; ○, one dose of GM_1; ●, four doses of GM_1; □, one dose of PA; ■, four doses of PA. In the group receiving four doses of PA, the number of mice was reduced to four due to the concurrent accidental deaths of two mice.

TABLE 2. Percentages of reticulocytes in different groups of mice infected with *P. berghei* on day 0, with or without previous (day −3) administration of PHZ

Group	% Reticulocytes[a]	
	Day 2	Day 7
PHZ-control (PHZ, no NFG)	51	39
PHZ-NFG (PHZ, NFG)	72	20
Infection control (no PHZ, no NFG)	1.9	18
NFG (no PHZ, NFG)	5.5	17

[a] Mean percentages of reticulocytes determined in blood smears for two mice per group.

to compensate for the loss (10). Parasitemia was recorded in mice infected 3 days after PHZ administration with (PHZ-NFG) or without (PHZ-control) two subsequent treatments with 7.5 mg of NFG/kg of body weight given i.p. For comparison, two groups of healthy mice (without PHZ) were infected and either treated with NFG in the same way or left untreated. The levels of reticulocytes (percent reticulocytes among the total number of erythrocytes) on day 2 and day 7 are given in Table 2; the levels of parasitemia are given in Fig. 4.

The effect of PHZ administration on reticulocytosis is evident. Without subsequent NFG treatment, the level of parasitemia rose earlier and much faster in PHZ-pretreated animals (Fig. 4B) than in infected healthy mice (Fig. 4A and previous experiments). During the first week p.i., the parasites in PHZ-pretreated mice (Fig. 4B) with high levels of reticulocytosis were as sensitive to NFG as those predominantly present in mature erythrocytes in healthy mice (Fig. 4A and previous experiments). The data in Table 2 indicate that malaria on its own causes considerable reticulocytosis, with or without NFG treatment, but that the very high level of PHZ-induced reticulocytosis is only maintained after a week in the absence of NFG treatment.

DISCUSSION

The experiments described here demonstrate that NFG initially has a strong inhibitory effect on *P. berghei* infections, as has been observed before (9), independent of the glycolipid used in the formation of the liposomes: it reduces the initial level of parasitemia drastically in a dose-dependent way (see experiment 2; Fig. 3B and D). Only one of the treated mice (treated with the lowest total dose used; 7.5 mg/kg of body weight in experiment 2; Fig. 3C) succumbed before day 14 p.i., while many of the untreated blood stage-inoculated mice (e.g., four of the six control mice in experiment 2; Fig. 3A and C) died before day 10, as usual. The cause of death in the latter group of mice is known to be almost always cerebral malaria. Hence, the results suggest that NFG can prevent mice from fatal cerebral malaria. This is a conclusion in retrospect, however, which has not been verified by direct pathological observation. Despite the positive effect of NFG on early parasitemia, none of the treated mice were cleared of the parasite, and all mice ultimately died, as it seems, from general malaria. In experiment 1 the treatment interval equaled the duration of the erythrocytic cycle of the parasite, which is 24 h for *P. berghei*. Lack of clearance could, in this case, have been caused by stage-dependent sensitivity, allowing some stages to escape treatment again and again. If this is the case, this should result in a shift in stage distribution in the treated animals compared to that in nontreated infected animals. Indeed, compared to the infections in the latter group, the percentage of ring stages at the time (hour of the day) of treatment increased in the treated animals from 14.7 to 23.5% after 2 days of treatment (day 3) and from 20.8 to 45.5% after 4 days of treatment (day 5). These differences are statistically significant (two-sample t test; $P < 0.01$), indicating that ring stages, if not completely insensitive, were at least less sensitive than non-ring stages, a phenomenon that has also been observed in experiments on the effect of NFG on *P. falciparum* in culture (12) and which is in line with the presumed mode of action of NFG (see the introduction).

In experiment 2, therefore, the treatment interval was shortened to 10 h so that it was no longer in phase with the erythrocytic cycle of the parasite, and the total dose given was increased 2.5 times to elongate the effective level of the drug in blood. Although the effect of these changes in treatment was clearly visible during the first week after treatment, the improved schedule did not result in elimination of the parasite. More disappointingly, repeat of the same treatment schedule at the time of a serious increase in the level of parasitemia (mostly day 9 or 10 p.i.; Table 1) had little (the group in experiment 2 treated with PA four times) or no effect on the level of parasitemia (Fig. 3).

Could this apparent insensitivity of the parasite to NFG after 1 week of infection be attributed to a change in the host cell? *P. berghei* is known to prefer reticulocytes (2, 6, 10). Only in the case of a shortage of these cells (which is the normal situation in healthy animals) will they invade young erythrocytes. As a result during the first week after blood inoculation most parasites are found in erythrocytes. With increasing levels

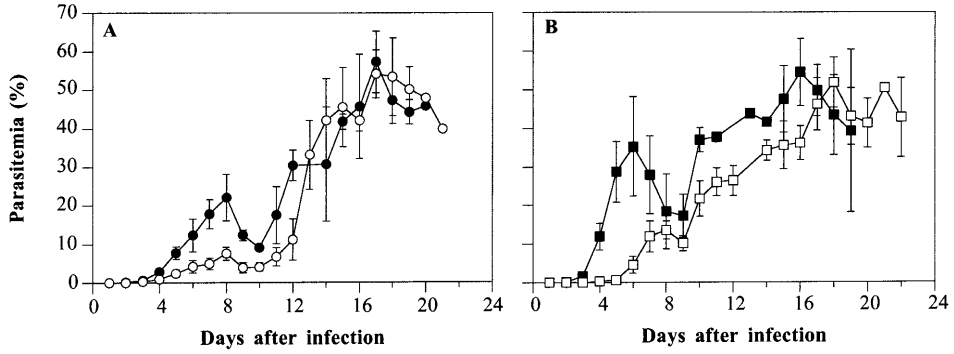

FIG. 4. Effect of PHZ treatment on the effectiveness of NFG in inhibiting parasite development (experiment 3). (A) Average levels of parasitemia for two groups of six mice that were infected on day 0; mice in one of the groups (open symbols) were treated with 7.5 mg of NFG/kg of body weight i.p. on day 1 and on day 2. (B) Average levels of parasitemia for two groups of six mice that received 50 mg of PHZ/kg of body weight 3 days before infection. ●, infection control; ○, NFG; ■, PHZ control; □, PHZ-NFG treatment.

of parasitemia even young erythrocytes are soon no longer sufficiently available, and as a result an initial rise in the level of parasitemia halts at about 20% and then tends to decline temporarily, as is seen in the infected control groups in experiments 1, 2, and 3 (Fig. 1, 2, and 4A). After a few days parasite-induced reticulocytosis provides the parasite with its preferred host cell, and from then the level of parasitemia rises very rapidly to values of over 50% (Fig. 3B and D and 4A) and most parasites are found in reticulocytes. If NFG were able to form stable channels in the weakened membranes of parasite-infected mature erythrocytes only and not in reticulocytes even when the reticulocytes are infected with *P. berghei* schizonts, this could explain the effect of NFG during the first week of infection and its absence during the later times of infection. If this were true, artificially induced pronounced reticulocytosis during the first week of infection should abolish the initial effect of NFG. In experiment 3, therefore, animals were pretreated with PHZ 3 days before infection, resulting in severe reticulocytosis, which was much higher than that induced after a week by the malaria parasites themselves (Table 2). Even so, the effect of NFG on the level of parasitemia during the first week of infection was not diminished, although the level of parasitemia, as expected, rose much faster and higher in the PHZ-pretreated control group. Again, however, the effect was only temporary.

In conclusion, we are as yet unable to explain the sharp difference in activity of NFG against malaria parasites during initial infection compared to that during the later times of infection. The same phenomenon was seen in *P. berghei*-infected BALB/c mice treated with two other malaria parasite inhibitors with profoundly different modes of action, HPMPA, an inhibitor of viral and eukaryotic DNA polymerases (3), and the presumed topoisomerase II inhibitor BQQ, which were administered to groups of mice in the same experiments described here (data not shown). These two inhibitors act on the parasites themselves. HPMPA is an inhibitor of the exonuclease moiety of the plasmodial DNA polymerase delta enzyme (15), and BQQ is assumed to inhibit the DNA relaxation activity of the DNA topoisomerase II during replication and transcription. In contrast, NFG is an erythrocyte membrane-bound compound that, most likely, does not act on the parasite itself because it is inserted into the host cell membrane (see introduction). For that reason it is highly improbable that in all our experiments the lack of effect of NFG during the later times of infection should be the result of selection of an NFG-resistant mutant, nor can the occurrence of this phenomenon be attributed to multidrug resistance as a result of changes in the membrane of the parasite itself.

Whatever the explanation might be, the experiments indicate that NFG is able to inhibit the growth of malaria parasites in vivo at concentrations that are well tolerated by the host. Therefore, and because of its unique mode of action compared to those of existing and candidate antimalarial agents, its potential as an antiplasmodial drug is worth further exploration.

ACKNOWLEDGMENTS

The present investigations were carried out under the auspices of The Netherlands Foundation of Chemical Research (SON) and with financial support from The Netherlands Organization for Scientific Research (NWO).

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