Stage-Selective Inhibition of Rodent Malaria by Cyclosporine

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The relative susceptibility of different developmental stages of *Plasmodium berghei* to cyclosporine was investigated *in vivo*. Within 12 h of receiving a single 25-mg/kg (body weight) dose of cyclosporine, mice with patent *P. berghei* infections uniformly exhibited a rapid fall in asexual parasite stages. Initially, ring forms and mature schizonts disappeared. Subsequently, trophozoites disappeared between 21 and 24 h, whereas gametocytes persisted for 36 h. In contrast, when cyclosporine was administered to mice 1 day before inoculation (100 mg/kg) with *P. berghei* sporozoites and for 2 consecutive days after inoculation (25 mg/kg), infections developed normally. When mice with patent infections were placed on prolonged cyclosporine therapy (25 mg/kg per day), parasitemia initially disappeared but often recrudesced. Recrudescence parasites were frequently resistant to cyclosporine (Cs^r). The Cs^r phenotype remained stable after serial passage of parasites in mice and after transmission through *Anopheles stephensi* mosquitoes, in which the capacity to produce oocysts was reduced. When infections of untreated mice were initiated with equal numbers of Cs^r and cyclosporine-susceptible (Cs^s) parasites and then carried through two serial cycles of mosquito-to-mouse transmission without cyclosporine treatment, the Cs^r phenotype was lost. The results indicate that cyclosporine selectively inhibits asexual blood stages of *P. berghei* and favors the emergence of Cs^r parasites with diminished infectivity for mosquitoes.

Recent studies (3, 5, 8) show that the cyclic undecapeptide cyclosporine has a potent antiparasitodal activity which is unrelated to the better known suppressive action of the drug on mammalian T-lymphocyte functions (1, 10). In mice infected with blood stages of *Plasmodium berghei* (5), *Plasmodium yoelii* (5), or *Plasmodium chabaudi* (8) and in owl monkeys infected with *Plasmodium falciparum* (3), normally rising levels of parasitemia are aborted and become undetectable after appropriate parenteral treatment with cyclosporine. Furthermore, when mice are inoculated with infectious blood stages of *P. berghei* or *P. yoelii*, three to four daily subcutaneous 25-mg/kg (body weight) doses of cyclosporine (initiated at the time of the infective inoculation) prevent the development of malaria (5). In both mice and monkeys, it appears that the earliest detectable fall in the level of parasitemia due to cyclosporine treatment coincides with a selective depletion of circulating ring forms (5). Moreover, in both species parasitemias "cured" by treatment with cyclosporine often recrudescence several days later and tend to be resistant to further cyclosporine treatment (5).

These observations formed the basis for the present study, in which the major aims were (i) to identify the cyclosporine-susceptible (Cs^s) stage(s) in the *P. berghei* life cycle and (ii) to examine and compare the stability and biologic properties of the cyclosporine-resistant (Cs^r) phenotype with those of the Cs^s phenotype.

MATERIALS AND METHODS

Plasmodia. *P. berghei* NK-65 (Cs^s) was obtained from G. Spitalny, Trudeau Institute, Saranac Lake, N.Y. Ampoules

of stock parasites (parasitized mouse erythrocytes [PRBC] in Alsever solution containing 10% glycerin) were stored at -70°C and were thawed and passaged once in mice before use in experiments. For selected experiments, the parasites were passed sequentially from mouse to mouse by *Anopheles stephensi* or syringe transfer of infected blood.

A Cs^r parasite population was obtained from mice infected with Cs^s *P. berghei* and subsequently treated with cyclosporine (25 mg/kg per day) through day 24 of infection, as described previously by Nickell et al. (5). The Cs^r parasites were passaged once through *A. stephensi* and twice through mice before being used to prepare a frozen stock.

Mosquitoes and mice. A mutant eye (*rosy*) strain of *A. stephensi* (4) (obtained from R. Sakai, University of Maryland International Center for Medical Research and Training, Lahore, Pakistan) was used in transmission experiments. Outbred 6- to 8-week-old female albino mice served as the experimental hosts.

Mosquito transmission. Infected mice with mature circulating gametocytes were restrained for 12 h in cages of adult *A. stephensi* in a 19°C incubator. Fully engorged females were separated and held at 19°C for 9 to 11 days for quantitation of oocysts or for 18 to 24 days when used as a source of sporozoites. Transmission of parasites to mice was accomplished either by allowing infected mosquitoes to feed (as described above) or by intravenous (i.v.) injection of 0.2 ml of a sporozoite suspension. The latter was prepared by triturating salivary glands in saline and subjecting the resulting suspension to low-speed centrifugation (50 × g) to remove debris.

Parasitemia. Parasitemia levels were determined from Giemsa-stained blood smears. The number of PRBC in 200 RBC was determined if the level of parasitemia was 1% or greater. For parasitemia levels less than 1%, the number of PRBC in a number of microscopic fields known to contain 10⁴ RBC was determined. For experiments in which individual asexual stages or gametocytes were enumerated, 500

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FIG. 1. Course of asexual parasitemia after i.v. injection of 10^6 PRBC of Cs^s or Cs^r *P. berghei* and the effects of two subcutaneous injections of cyclosporine (CS) (25 mg/kg per injection) delivered on days 5 and 6. There were six mice per group through day 5 and three per group thereafter.

RBC or microscope fields containing a total of about 2×10^4 RBC were examined (for >1 or $<1\%$ parasitemias, respectively).

Exflagellation. Samples (0.05 ml) of tail blood were diluted 1:25 in normal mouse serum. A portion of each sample was placed in a hemacytometer, exposed for 30 s to 95% CO₂, and held at 22 to 24°C for 10 min. The number of exflagellating bodies in 0.2 mm³ was then counted by phase-contrast microscopy.

Oocysts. Mosquito midguts were dissected in normal saline on microscope slides and flattened with a cover slip. Their contents of oocysts were counted by using a phase-contrast microscope.

Cyclosporine and chloroquine treatments. Cyclosporine (provided by J. Borel, Sandoz Ltd., Basel, Switzerland) was dissolved in Emulphor El 620 (donated by GAF Corp., New York, N.Y.) at a ratio of 100 mg/2 ml and further diluted with distilled water to achieve the desired drug concentration. Chloroquine hydrochloride (Winthrop Laboratories, New York, N.Y.) was dissolved in saline to a concentration of 300 mg/ml. Both drugs were administered subcutaneously in the flank.

RESULTS

Characteristics of Cs^s and Cs^r parasites. Infections initiated i.v. with 10^6 PRBC (either phenotype) progressed at approximately the same rate and were uniformly fatal (Fig. 1). Treatment with cyclosporine (25 mg/kg) on days 5 and 6, when parasitemia levels averaged about 1%, did not affect the course of Cs^r infections, whereas Cs^s infections were reduced rapidly to undetectable levels ($<0.01\%$). The latter recrudesced in all mice after 5 to 8 days and were progressive and lethal in most (data not shown). On the basis of these findings, sensitivity to cyclosporine was defined as a 100-fold or greater fall in the level of parasitemia within 48 h after administration of the first drug dose.

In an attempt to assess the stability of the Cs^r phenotype in the absence of cyclosporine, Cs^r parasites were subjected to 10 consecutive passages in mice (by blood inoculation)

which were not treated with cyclosporine or to five mosquito-to-mouse transmission cycles. At every passage, when levels of parasitemia averaged about 5%, a tail blood sample was collected from each recipient mouse and inoculated into two or three normal indicator mice. Developing parasitemias of indicator mice were tested for cyclosporine sensitivity as defined above. In all instances, the Cs^r phenotype was retained.

Effect of cyclosporine on asexual erythrocytic-stage parasites. To determine the dynamics of cyclosporine-mediated parasite clearance, patently infected mice were given a single dose (25 mg/kg) of cyclosporine and observed for 24 h (Fig. 2). The disappearance of individual plasmodial stages from blood was asynchronous. Ring forms and schizonts were not observed after h 9 posttreatment. A much slower waning of trophozoites occurred during the next 8 h.

The transient disappearance of schizonts in treated mice at h 1 after treatment is not considered significant. A transient disappearance of these relatively infrequent forms also occurred in untreated controls (12 h); this may reflect a degree of synchronization of infection. The continued inability to find schizonts in the cyclosporine-treated group for six sequential intervals from 12 through 24 h is considered significant.

Effects of cyclosporine on sporozoites, exoerythrocytic stages, and gametocytes. To ensure the presence of cyclosporine during liver-stage infection, mice were given the drug at 24 h (100 mg/kg) and 1 h (25 mg/kg) before receiving sporozoites i.v. and at 24 h (25 mg/kg) after sporozoite inoculation. Based on previous results (5) which demonstrated that the in vivo anti-erythrocytic-stage action of cyclosporine is of less than 48 h duration, cyclosporine treatment was stopped at 24 h of liver infection to allow levels to diminish to below the threshold required for anti-erythrocytic-stage effect by day 5, the earliest expected emergence of erythrocytic-stage parasites. Under this regimen, cyclosporine did not prevent the development of patent infections, although it caused an increase in the interval required to reach 1% parasitemia (Table 1).

The cyclosporine susceptibility of gametocytes was assessed at 9 days postinfection, when they could be easily enumerated in thin blood smears (Fig. 3 and 4). For Cs^r parasites, treatment with cyclosporine did not reduce the numbers of gametocytes or exflagellating units. The infection rate and the number of oocysts per infected mosquito did not differ significantly from those for untreated controls. In contrast, levels of circulating gametocytes in Cs^s-parasite-infected mice began to decline 12 h after drug treatment. Gametocytes persisted at significantly reduced levels

FIG. 2. Progress of established (5-day) Cs^s-asexual-stage parasitemias over a 24-h interval in untreated mice and mice injected with 25 mg of cyclosporine (CS) per kg at h 0. There were three mice per group per time point.

TABLE 1. Effects of cyclosporine on sporozoite-initiated C^s infection

Treatment	No. of mice infected (n = 10)	Mean time (days) to 1% PRBC
None	6	6.17
Cyclosporine ^a	7	7.90 ^b

^a Administered 24 h (100 mg/kg) and 1 h (25 mg/kg) before sporozoite injection and 1 day (25 mg/kg) after sporozoite injection.

^b $P < 0.01$, Student's t test.

through 48 h after the initiation of cyclosporine treatment. Infectivity for mosquitoes continued through 36 h.

Disassociation of cyclosporine resistance and chloroquine resistance. Patent infections produced with either C^s or C^r parasites were compared for their sensitivity to chloroquine. At a 30-mg/kg dose, parasites of both phenotypes showed equal susceptibility to chloroquine (data not shown).

Competence and competition of C^s and C^r parasites. During the course of our studies, C^r parasites were more difficult to maintain by mosquito passage than those of the parental phenotype. To determine whether C^r parasites were less competent in the mosquito than C^s parasites, separate groups of mice were infected with blood-stage parasites of each phenotype. Nine days later, half the animals in each group were given the first of two daily doses (25 mg/kg) of cyclosporine. Beginning at day 9 of infection and again after two consecutive 24-h periods, individual batches of 100 to 150 *A. stephensi* were fed on drug-treated and untreated mice from each group. Oocyst numbers were subsequently determined from dissections of mosquitoes. Despite similar levels of gametocytes in C^s - and C^r -parasite-infected untreated mice (Fig. 3 and 4), significantly

FIG. 3. Effects on sexual stages of two sequential 25-mg/kg injections of cyclosporine (CS) delivered at days 9 and 10 of infections. The 0-h point was immediately before cyclosporine injection. Samples from five mice were evaluated per time point.

FIG. 4. Effects of cyclosporine (CS) treatment on transmission of infection to *A. stephensi*. The 0-h point was immediately before the first cyclosporine injection and was day 9 after infection. This experiment used mice challenged with the same inoculum as that used for the experiment for which results are presented in Fig. 3. Five mice were used per time point, and each mouse was separately exposed to between 100 and 150 female *A. stephensi*. Between days 9 and 11 after feeding, a minimum of 25 mosquitoes from each mouse feed were dissected to determine numbers of oocysts.

fewer oocysts were observed in mosquitoes fed on mice infected with C^r parasites. The results demonstrate that C^r parasites are less competent than C^s parasites to develop in mosquitoes.

The comparative fitness of other developmental stages of the two parasite lines was tested. First, the relative capacity of asexual blood stages to grow in mice was investigated. Separate groups of mice were inoculated with 10^6 PRBC of the C^s or C^r strain or with a mixture of 5×10^5 PRBC of each strain. The growth curves showed nearly identical rates of parasitemia for the three groups (Fig. 5), indicating that when maintained separately, the two parasite lines are equally fit for development in mice.

For mice with mixed infections, further trials were conducted to determine whether C^r parasites constituted a large fraction of the parasitemia. It was reasoned that if a preponderance of C^s parasites was circulating in groups of such mice, cyclosporine injection would reduce parasitemia in proportion to the number of C^s parasites. In two separate trials (the results for one are shown in Fig. 5), this approach demonstrated the persistence of large numbers of C^r parasites.

To determine whether prolongation of asexual-stage infection would result in the predominance of C^s parasites, plasmodia were recovered from mice with patent mixed infections and subjected to four additional serial passages. Between 8 and 10 days after each passage, blood from three

FIG. 5. Patterns of parasitemia in separate groups of mice infected with Cs^s , Cs^r , or Cs^s plus Cs^r parasites. Patterns are for either untreated mice or treated mice during the 4 days immediately after the initiation of cyclosporine (CS) treatment regimens. Treatment was initiated for separate groups at day 5, 10, or 15 of infections. There were three mice per time point, and cyclosporine injections of 25 mg/kg were delivered at the indicated intervals. All Cs^r -parasite-infected mice treated with cyclosporine on day 15 died by day 18.

infected (donor) mice was subinoculated into three normal (recipient) mice, and the sensitivity of the resulting parasitemia to cyclosporine was measured. At each passage the parasites were resistant to cyclosporine, thus demonstrating that Cs^r parasites are well suited for survival in the intermediate host.

A different result was obtained when parasite populations from two donors with mixed infections were subjected to four consecutive mosquito-to-mouse transmission cycles. After two cycles, the transmitted infections were uniformly sensitive to cyclosporine treatment.

DISCUSSION

Cyclosporine has been shown to interrupt erythrocytic schizogony in *P. berghei* infection of mice but not to affect exoerythrocytic schizogony or sexual erythrocytic stages. The effect of cyclosporine on erythrocytic schizogony was selective, with more rapid disappearance of rings and schizonts than trophozoites. The pattern of parasite-stage-specific inhibition by cyclosporine is similar to that which follows chloroquine treatment. However, the complete susceptibility of Cs^r parasites to treatment with chloroquine suggests that the antiparasitic mechanisms of the drugs differ.

The activity of cyclosporine on sporozoites and early liver stages was investigated under conditions in which drug dosages and treatment frequency exceeded the threshold

required to clear asexual-erythrocytic-stage parasitemia. The results support three views: (i) sporozoites are innately insensitive to cyclosporine, (ii) cyclosporine does not bind in effective concentration to circulating sporozoites, or (iii) the environment of the liver parenchymal cell neutralizes the action of sporozoite-associated cyclosporine. The failure of cyclosporine pressure to arrest early exoerythrocytic schizogony suggests that either the liver stages of *P. berghei* are innately resistant or that cyclosporine is incapable of reaching parasites within the liver.

Although cyclosporine treatment of Cs^s infection resulted in a significant decrease in circulating gametocytes, the rate of clearance was much slower for gametocytes than for asexual erythrocytic forms. The gametocytes which persisted remained infective to mosquitoes. The decrease in the level of gametocytemia may reflect the normal attrition of senescent forms and their lack of replacement because of interruption of the asexual cycle. The successful establishment of mosquito infection in the face of ongoing cyclosporine treatment demonstrates that the levels of cyclosporine imbibed with blood meals does not ablate syngamy or the progress of the initial stages of mosquito infection. Rather, the number of gametocytes ingested may have been markedly reduced.

Rosario et al. (7) have demonstrated that the acquisition of drug resistance by plasmodial parasites may be offset by alterations in the biological competence of parasite populations. For example, pyrimethamine resistance in *P. chabaudi* is associated with a biological debit: the drug-resistant parasites are less well fit to complete the life cycle than are wild-type parasites. On the other hand, chloroquine resistance (7) provides *P. chabaudi* with a distinct advantage under similar conditions, the acquisition of drug resistance being linked to an improved capacity to complete the life cycle. Our difficulty in passing Cs^r *P. berghei* through mosquitoes and the demonstration that mosquitoes fed Cs^r *P. berghei* developed fewer oocysts than those fed similar numbers of Cs^s *P. berghei* suggest that the acquisition of the Cs^r trait is linked to changes which place resistant parasites at a biological disadvantage. When this proposition was tested by admixing Cs^s and Cs^r parasite populations, the Cs^r trait was shown to reduce parasite competence. This was expressed as a lessened capacity of Cs^r parasites to complete the life cycle.

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