Falciparum Malaria: Transmission to the Gibbon by Anopheles balabacensis

Abstract. *The splenectomized gibbon* (Hylobates lar) *is susceptible to sporozoite-induced infection by sporozoites of* Plasmodium falciparum. *Two gibbons inoculated with sporozoites of* P. falciparum *from* Anopheles balabacensis *fed on humans with falciparum malaria developed parasitemia 48 and 46 days after infection.*

The splenectomized gibbon (*Hylobates lar*) is susceptible to blood-induced infection with *Plasmodium falciparum* (*1*). We now report that the splenectomized gibbon can also support the exoerythrocytic stage of *P. falciparum*. Thus, for immunologic and chemotherapeutic studies of falciparum malaria, its potential value as a laboratory host is extended. Until now, the splenectomized chimpanzee (*Pan satyrus*) was the only higher primate, aside from man, known to be susceptible to infection by sporozoites of *P. falciparum* (*2*).

Two gibbons (1 to 2 years old; body weight, 1.75 to 2.75 kg) were inoculated with sporozoites of *P. falciparum* from *Anopheles balabacensis* which had been reared in the laboratory and fed on malarious patients from Saraburi Province, Thailand. After the gibbons had been screened for natural malarial infections and had become acclimatized to colony conditions, they were splenectomized. One animal (S-1) was treated for possible latent malarial infections with chloroquine and primaquine as described previously (*1*), while the other animal (P-10) was untreated. Malarial parasites were seen in the blood of neither animal either before splenectomy or prior to inoculation. During the course of the experiment the gibbons were housed in quarters free of mosquitoes.

Two months after being splenectomized, gibbon P-10 was exposed to four *A. balabacensis* which were part of a group fed 16 days earlier on an adult patient who had, at the time of the feed, 1687 gametocytes per microliter of blood. Sporozoites were seen in the glands of other mosquitoes of this group dissected on day 15. As only one of the four mosquitoes fed to engorgement, all were ground up in 50 percent human serum (with isotonic saline as the diluent), and P-10 was inoculated intramuscularly with the supernatant from this suspension. Characteristic ring forms of *P. falciparum* were seen in the blood of this animal after a prepatent period of 47 days. Asexual parasites were continuously seen in P-10 until the end of the observation period on day 83. On day 64 a peak count of 3271 trophozoites per 500 white blood cells was observed (approximately 62,500 per microliter). The first gametocytes were seen on day 62 and were observed for 16 days thereafter. The maximum count of 50 gametocytes per 500 white blood cells (1000 per microliter) was observed on day 65. Of the asexual forms, only trophozoites were seen, while all of the gametocytes observed were young and either rounded or oat-shaped. Mature, sausage-shaped gametocytes were not seen.

Four months after it was splenectomized, gibbon S-1 was inoculated in the above manner with a suspension of 12 mosquitoes from a group of *A. balabacensis* fed 17 days earlier on a patient with 4536 *P. falciparum* gametocytes per microliter of blood. Sporozoites were seen in the salivary glands of other mosquitoes from this group on the day of inoculation. After a prepatent period of 45 days, small ring forms were seen in the blood of this animal. Gibbon S-1 continued to circulate asexual forms until the end of the observation period on day 73. Maximum counts of 130, 122, and 115 trophozoites were observed on days 50, 65, and 67, respectively. Asexual forms other than trophozoites were not seen, nor were gametocytes observed in the blood of this animal.

The prepatent period in these gibbons was considerably longer than that reported for humans infected with this parasite by way of mosquito bites; the medians in 161 cases ranged from 9 to 13 days (*3*). This longer period may be related to the relatively small number of sporozoites in the inoculum, to partial susceptibility of the gibbon to this parasite, or to an extended period of exoerythrocytic development of the parasite in this host species.

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Drug-Induced Teratogenesis in vitro: Inhibition of Calcification by Different Tetracyclines

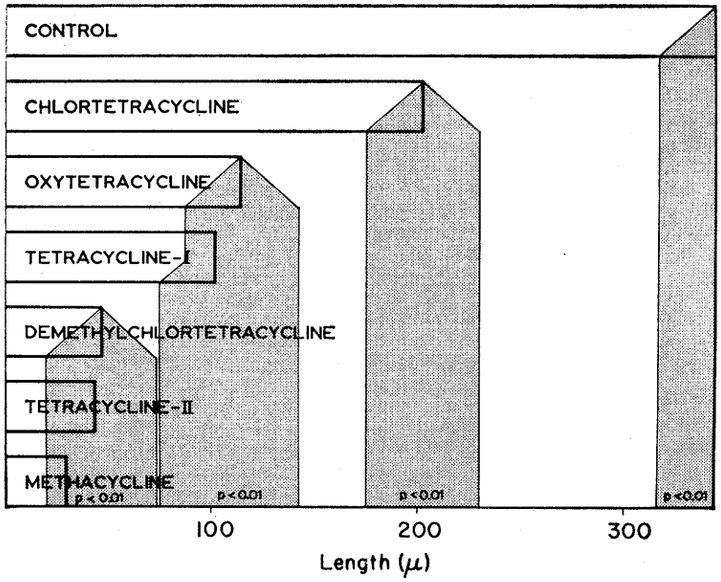
Abstract. *Inhibition of calcification in embryonic bone rudiments was studied in the presence of several tetracyclines at three different concentrations. Different criteria for calcification and different concentrations of tetracyclines yielded parallel results and showed significant differences in the inhibitory action of the various compounds. The clear-cut results indicate that the test-system that was developed may be useful for the comparison of various teratogens under simplified controllable conditions.*

Recent experience in testing the teratogenicity of drugs has shown that the conclusiveness of classic animal experiments is limited. Consequently, only large series performed on a number of different species can give reliable information. It was thought useful, therefore, to explore the possibilities of performing such tests in simplified model-systems in vitro, where side-effects could be determined more exactly. The tetracycline antibiotics were chosen for these tests. Both clinical experience and experimental results have shown that these drugs interfere with calcification of embryonic bones, in which the drug is selectively incorporated (*1*). The effect can be demonstrated and quantitated in organotypic cultures of embryonic bones (*2*), and hence this model-system was employed for certain comparative experiments. A variety of tetracyclines that are chemically closely related are in clinical use, and earlier experiments have indicated that

their effects on osteogenesis may be different (*3*). Consequently, six different commercial tetracyclines were employed in these in vitro experiments and their inhibitory effects compared.

Ulnar bone rudiments of 17-day mouse embryos were aseptically removed and cultured on lens paper placed on stainless steel grids at the medium-gas interface. A chemically defined medium was used without addition of serum or embryo extract. The medium was basically that developed by Biggers (*4*), but addition of ascorbic acid in a concentration of 5 μg/ml proved to be of major importance for calcification (*5*). Total volume of medium was 100 times that of bones, and the medium was not changed during cultivation.

Tetracycline samples were obtained in purified form as hydrochlorides, and six different compounds were tested (*6*). Since the therapeutic concentra-



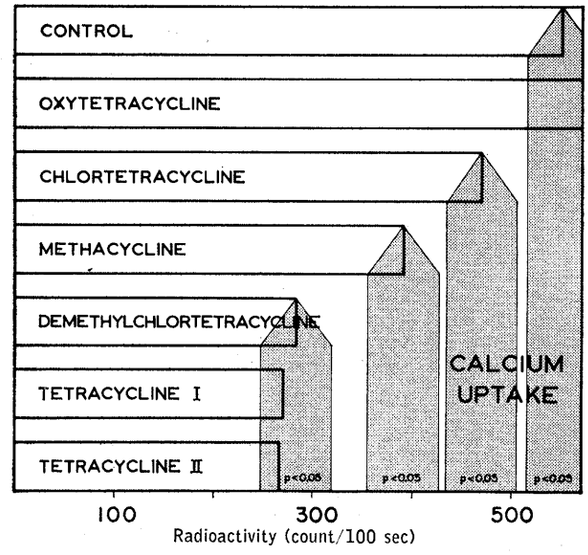
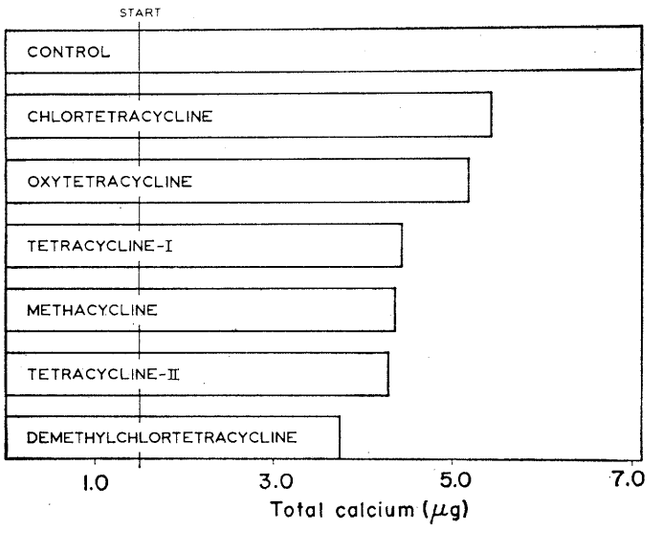


Fig. 1 (above left). Growth of calcified zone of embryonic bone rudiments cultivated in the presence of different tetracyclines at a concentration of 5 μg/ml. Shaded columns indicate the 99-percent significance level of differences calculated from growth of individual bones. Fig. 2 (above right). Uptake of labeled calcium (count/100 sec) by bones cultivated for 5 days in the presence of different tetracyclines (concentration, 5 μg/ml). Fig. 3 (below). Total calcium of bones indicated in Fig. 1. The starting value was determined from 10 randomly selected rudiments of the original pool of 80 bones. Fig. 4 (right). Results of inhibition of calcification in the presence of six different tetracyclines at three concentrations. Bars indicate the percentage of inhibition obtained according to the different criteria and compared with changes in parallel control cultures. (*A*) Length of the mineralized zone; (*B*) total calcium; (*C*) uptake of labeled calcium. Numbers indicate the percentage of inhibition based on the three different criteria.



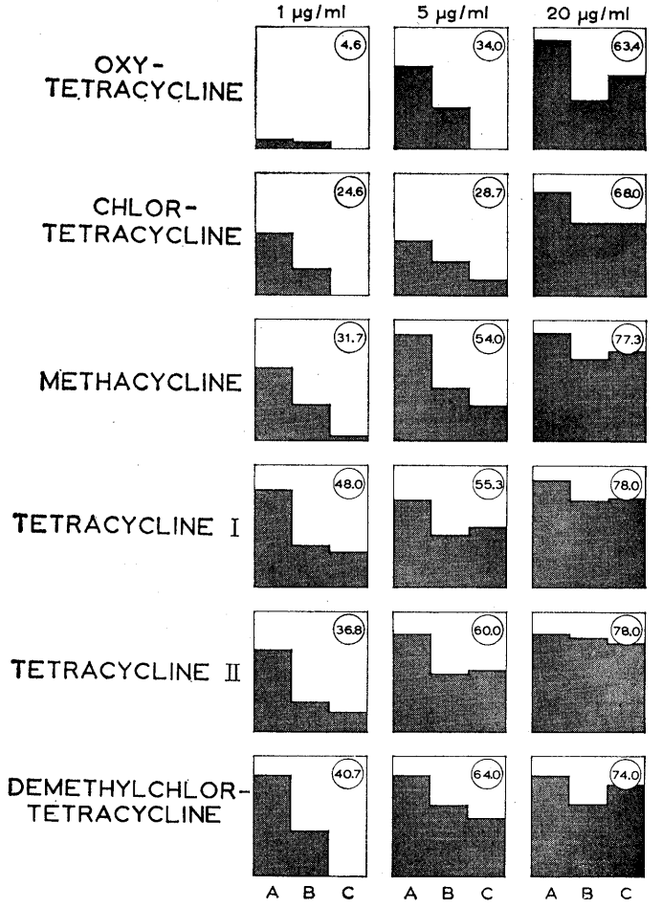


Table 1. Number of bone rudiments in different series of experiments. Six different tetracyclines were used at three concentrations each.

Conc. of tetracyclines (μg/ml)	No. tested at start of exp. for Ca + length	No. bone rudiments tested after 10 days for			
		Ca + length		Ca uptake	
		Controls	Tetracyclines	Controls	Tetracyclines
1	10	5	6 × 5	5	6 × 5
5	10	10	6 × 10	10	6 × 10
20	10	5	6 × 5	5	6 × 5
Total No.	30	20	120	20	120

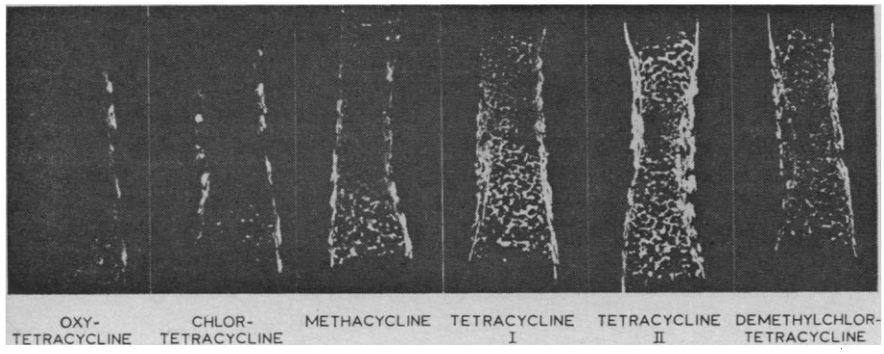


Fig. 5. Pseudoquantitative demonstration of the amount of fluorophore in bones treated for 4 days with different tetracyclines at a concentration of 5 μg/ml. Sections of standard thickness were photographed and reproduced under standard conditions.

tion of these drugs in serum is known to be approximately 1 to 5 μg/ml (*7*), three concentrations were tested—1.0, 5.0, and 20 μg/ml. The dry sample was first dissolved in distilled water to make a stock solution of 100 μg/ml, and this was used in making the final dilution in the medium.

Three criteria were adopted for studying the calcification of the rudiments: (i) Length of the mineralized zone was measured. This could be done easily from camera lucida drawings made at magnifications of × 50. Individual bones were drawn at the beginning of the culture period and again 10 days later. (ii) Total calcium was determined by flame photometry from five or ten pooled bones. This was performed with ten bones that represented the starting material in each series and again with bones that had been cultivated for 10 days. (iii) The uptake of labeled calcium was determined in individual bones that were cultivated in the presence of calcium-45 (0.02 μc/ml) (*8*). After 5 days of cultivation, bones were rinsed in distilled water, dried for 1 hour in an incubator at 37°C and treated with "Hyamine" hydroxide (*9*) for 10 minutes. Radioactivity was determined in a liquid scintillation spectrometer. The number of bone rudiments treated in the different series is given in Table 1.

Figures 1 to 3 show results of quantitative determinations of calcification in the presence of the various tetracyclines in concentrations of 5 μg/ml. Statistical analysis indicates significant differences between the series in elongation of the calcified zone and in rate of uptake of radiocalcium. The third parameter, determination of total calcium, gave very similar results, although statistical treatment of the pooled samples was not possible. All three criteria indicate that two of the compounds tested, oxytetracycline and chlortetracycline, have a less pronounced inhibitory effect than the other four tetracyclines.

Similar experiments were performed employing concentrations of 1 and 20 μg/ml. All results are summarized in Fig. 4, and they seem to justify two main conclusions: (i) inhibition of calcification is concentration-dependent, and (ii) the same differences between various tetracyclines can be demonstrated at different concentrations. The three criteria gave very similar results, although the simplest of them, length of the calcified zone, seemed to be the most sensitive. The tetracycline fluorophore was examined pseudoquantitatively in bones treated with different tetracyclines. Sections of standard thickness were prepared from fixed undecalcified rudiments and photographed in ultraviolet light under standard conditions. Figure 5 shows these fluorograms, and it can be concluded that the two tetracyclines with weakest inhibitory effect produce less fluorophore in the bones.

In order to evaluate the adequacy of this test system, the results obtained should be compared with earlier tests with the same drugs. Only a few comparative studies with tetracyclines have been reported; in two of these, discoloration of teeth and fluorescence in the enamel have been compared pseudoquantitatively (*10*), and in three others the effect of different tetracyclines on chick embryos has been quantitated (weight of embryo, incidence of malformations, and length of long bones) (*3*). In the former studies, oxytetracycline gave the weakest discoloration and fluorescence when compared with demethylchlortetracycline and tetracycline; in the latter, the results indicated that demethylchlortetracycline is the most potent inhibitor, whereas both chlortetracycline and oxytetracycline have less harmful side-effects on the developing embryos.

My results seem to be in good accordance with these in vivo observations, and it thus appears that the organotypic model-system employed may be a useful tool in such comparative studies. It may even have certain advantages as compared with in vivo methods: the tissue culture conditions can be kept well standardized, no unknown compounds are present in the chemically defined medium, calcification can be followed by exact measurements, and the test is relatively rapid. Future work will show to what extent this type of test-system can be applied to other drugs with teratogenic activity.

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6. Samples were obtained through the courtesy of the Lederle Laboratories, Cyanamide of Great Britain, Ltd., London, and the Pfizer Corp., Brussels. The following drugs were tested: tetracycline I ("Tetracyn"), tetracycline II ("Achromycin"), oxytetracycline ("Terramycin"), chlortetracycline ("Aureomycin"), methylene hydroxytetracycline (methacycline) ("Rondomycin"), and demethylchlortetracycline ("Ledermycin").
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DNA Synthesis and Differentiation in Embryonic Kidney Mesenchyme in vitro

Abstract. *Mitosis accompanies the differentiation of embryonic metanephrogenic mesenchyme in vitro. Inhibition of mitosis by 5-fluorodeoxyuridine, but not by x-rays, is associated with inhibition of differentiation. The difference may be due to the effect of 5-fluorodeoxyuridine on DNA synthesis.*

Inhibition of DNA synthesis by 5-fluorodeoxyuridine reportedly inhibits differentiation (elongation) of nondividing plant cells (*1*). 5-Fluorodeoxyuridine has an inhibitory effect on the differentiation of embryonic metanephrogenic mesenchyme in vitro, which may also be independent of cell division.

5-Fluorodeoxyuridine (FUDR) specifically inhibits thymidylate synthetase (*2*, *3*), thus blocking DNA synthesis (*4–6*) and, consequently, inhibiting mitosis (*6*) and cell division (*4*, *5*). To determine whether inhibition of differentiation by FUDR was caused by inhibition of DNA synthesis or mitosis or both, I tried to distinguish between these alternatives by comparing the effect of mitotic inhibition by x-rays with the effect of treatment with FUDR.

Mesenchyme from the metanephric kidney of the mouse embryo differentiates in vitro in response to inductive stimulation from embryonic dorsal spinal cord (*7*). By 24 hours of culture most of the mesenchymal tissue is arranged in whorls, containing many cells in mitosis, which, on further culture, gradually develop into tubules.

Metanephric kidneys and dorsal spinal cords were isolated from 11-day-old mouse embryos [C×C_3H(HeAu)] by methods previously described (*7*). The kidney rudiments were incubated for 4 to 5 minutes in a 3 percent trypsin solution containing 3 parts trypsin (1:250 Difco) to one part pancreatin (Viokase, Viobin Corp.) dissolved in calcium- and magnesium-free Tyrode's solution. Mesenchyme was then separated from the ureteric bud by flushing the kidney into and out of a micropipette (*7*). Mesenchyme from two to three kidney rudiments was fused to a piece of dorsal spinal cord, cultured in a filter-well assembly (*8*) in a Grobstein dish containing 1.5 ml of medium, and maintained in a humidified incubator gassed with 5 percent CO_2 in air at 37°C. 5-Fluorodeoxyuridine and $10^{-4}M$ uridine were added to medium containing 1 percent horse serum and 50 units of penicillin-streptomycin per milliliter in Eagle's basal medium (Medium A.) Irradiated tissues were cultured in medium containing 3 percent embryo extract, 10 percent horse serum and 50 units of penicillin-streptomycin per milliliter in Eagle's basal medium (Medium B). Both media support differentiation, but growth is more extensive and survival longer in the more richly supplemented medium. Mesenchyme to be irradiated was maintained in a culture dish containing 0.5 ml of medium placed in a glass container filled with 5 percent CO_2 in air. X-rays were administered at 140 kv (peak) and 5 ma with an inherent filtration of ½ mm of aluminum at a target distance of 30 cm in single-dose treatments of 1500 r at a rate of 55 r/min. Dosage was monitored with a Victoreen dosimeter.

To determine the effects of FUDR and x-ray treatments on DNA synthesis, incorporation of tritiated deoxycytidine was studied by autoradiography. Tissues were incubated for 1 hour in medium containing 10 μc of tritiated deoxycytidine per milliliter (Schwarz BioResearch, Inc., 2.4 c/mmole), washed twice in medium without labeled precursor, fixed in Carnoy's for 40 minutes, embedded, and sectioned at 4 μ. Alternating strips of the paraffin ribbon containing four or five sections of tissue were placed on three slides. Tissues on two slides were hydrolyzed for 4 hours at 37°C: one in 0.3 mg of deoxyribonuclease (Worthington) per milliliter in $4 \times 10^{-3}M$ $MgSO_4$, *p*H 7, the other in 0.3 mg of ribonuclease (Worthington) per milliliter, *p*H 7. The third slide was untreated. All slides were coated with Kodak AR-10 stripping film at 25°C, developed in D19b developer after 2 weeks of exposure, and stained with Delafield's hematoxylin. Treatment with ribonuclease caused no significant alteration in the pattern of labeling, whereas treatment with deoxyribonuclease removed virtually all the labeled material. For routine histological preparations, the cultures were fixed in Carnoy's, sectioned at 5 μ, and stained with Delafield's hematoxylin and eosin.

The effect of increasing concentrations of FUDR on differentiation is summarized in Table 1, experiment 1. Cultures grown in $10^{-6}M$ FUDR are indistinguishable from controls. Those grown in $10^{-5}M$ FUDR are retarded in development, and the tubules which are formed are smaller, are not as well shaped as those in controls, and contain fewer mitotic figures. Cultures survive for at least 24 hours in $10^{-4}M$ FUDR but show no signs of differentiation. No mitotic figures were observed at 24 hours.

Autoradiographs prepared from tissues cultured in medium containing $10^{-4}M$ FUDR to which tritiated deoxycytidine was added during the 4th to 5th hour of incubation show that treatment with FUDR greatly reduces, but never completely abolishes, uptake of labeled precursor in both the mesenchyme and dorsal spinal cord. It was impossible to quantitate the extent of the inhibition because there was no discernible regularity in the distribution of labeled cells throughout the cultures. Resistance to FUDR (*3*) and synthesis of "DNA-like material" during thymidineless growth (*4*) has been reported for other tissues, but the mechanisms involved are not clear.

The inhibitory effect of FUDR on differentiation was shown to be reversible in two ways. Cultures were grown in medium containing $10^{-4}M$ FUDR to which $10^{-4}M$ thymidine was added. Differentiation proceeded normally in this medium (Table 1, experiment 2). All media supplemented with FUDR contained $10^{-4}M$ uridine. Under modified culture conditions, in which differentiation can be inhibited with lower concentrations of FUDR, a hundredfold increase in the concentration of uridine