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Antimalarial Activities and Subacute Toxicity of RC-12, a 4-Amino-Substituted Pyrocatechol†

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RC-12 [1,2-dimethoxy-4-(bis-diethylaminoethyl)-amino-5-bromobenzene] was evaluated for prophylactic, radical curative, and suppressive activities against infections with *Plasmodium cynomolgi* and subacute toxicity in rhesus monkeys. Applied as a prophylactic agent, RC-12, administered in doses of 6.25 to 25.0 mg/kg daily throughout the incubation period, provided near-complete to complete protection against 10^5 to 10^6 times the minimum infective dose of sporozoites. Applied as a suppressive agent, daily doses of 100.0 mg of RC-12 per kg did not eradicate blood schizonts regularly; hence, the need for concomitant administration of a blood schizonticide, such as chloroquine, in assessments of radical curative activity. In such appraisals, daily doses of 6.25 to 25.0 mg of RC-12 per kg for 14 days, in combination with 2.5 mg of chloroquine per kg daily for 7 days, effected cure of 69 and 93% of established infections, respectively. The curative activity of RC-12 was related to the total dose and could be achieved with a regimen as brief as 4 days. With respect to outward expressions of toxicity, daily doses of 50.0 mg/kg or lower for 15 to 225 days evoked no reactions. Doses of 100.0 or 200.0 mg/kg, scheduled for 15 days, evoked convulsions and depression and were, respectively, lethal to 4 of 17 and 7 of 7 recipients. Doses of 25.0 mg/kg or lower evoked no discrete reactions. Doses of 50.0 mg/kg and higher evoked hepatomegaly, vacuolation of hepatocytes, and elevations of glutamic oxalacetic and glutamic pyruvic transferase activities in serum, reactions related in intensity to dose but not duration of dosage.

RC-12 [1,2-dimethoxy-4-(bis-diethylaminoethyl)-amino-5-bromobenzene] is a 4-amino-substituted pyrocatechol. Interest in compounds of this class as antimalarial agents dates back to 1926 when investigators at I. G. Farbenindustrie, Frankfurt am Main, described the synthesis of Dimeplasmin [1,2-dimethoxy-4-(bis-diethylaminoethyl)-aminobenzene] and its activities against infections with trophozoites of *Plasmodium relictum* in canaries (W. Schulemann and W. Kropp, Verfahren zur Darstellung von N-Dialkyl-aminoalkylderivaten aromatischer Aminooxy- und Diaminoverbindungen; German patent 499,826, 1930). Shortly thereafter, Dimeplasmin was evaluated for activity against both blood-induced and naturally acquired infections with *Plasmodium falciparum* and *Plasmodium vivax*, where it proved to be less effective than quinine in controlling parasitemia (11, 12, 43; personal communications to W.S. from Muhlens, 1928, and Sioli, 1929). At that point, those involved in these studies turned attention to the newly prepared Atebrin (quinacrine) (18).

In 1946, two of the authors of this report (W.S., who had been a major participant in the 1926 studies, and L.K.) undertook a reinvestigation of the antimalarial properties of the 4-amino-substituted pyrocatechols. This renewed interest stemmed from the attention then being given to the development of antimalarial drugs with capacity for radical cure (41) and from recently acquired knowledge which showed that: (i) the life cycles of various avian plasmodia in the vertebrate host were characterized by a primary tissue phase, an erythrocytic phase, and a secondary tissue phase (14–16), and (ii) these phases differed in susceptibility to attack by antimalarial drugs (7). W.S. and L.K. recognized that as infections with *P. relictum* had been manipulated in 1926, they would have identified agents active against the erythrocytic (blood) phase of infections, but not against either primary or secondary tissue phases. They further recognized that canaries inoculated with sporozoites of *Plasmodium cathemerium* had the potential of identifying agents active against the latter phases; hence, they used this avian malaria in their post-1946 studies. By 1960, they had shown that any one of four structurally related pyrocatechols, including Dimeplasmin and RC-12, administered either soon after sporozoite inoculation or at onset of parasitemia, prevented development of the secondary tissue stages of the above avian plasmodium and evolution of fatal disease. These accomplishments were unlike those resulting from administration of quinine, quinacrine, or chloroquine, but were like those resulting from application of pamaquine or primaquine. These demonstrations led one of the investigators (W.S.) to suggest to the World Health Organization, provider of support for his study, that an investigation of the capacity of one of the pyrocatechols to effect prophylaxis and radical cure of infections with *P. vivax* be undertaken. This suggestion led to the studies on the activity of RC-12 against infections with *Plasmodium cynomolgi* in rhesus monkeys encompassed in this report. RC-12 was selected for the investigation because its therapeutic index (ratio of toxic to therapeutic dose) in studies carried out in the canary was larger than that of any one of the other three chemically related agents. Infections with *P. cynomolgi* were selected

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for the evaluation because they are the biologic and chemotherapeutic replicas of infections with subtropical zone strains of *P. vivax* in humans and can be manipulated readily in the laboratory (34–36, 38).

This report brings together the results of two series of studies started in January 1965 and completed in December 1967. The first dealt with the capacities of RC-12 to protect rhesus monkeys against infections with sporozoites of *P. cynomolgi*, to cure established infections, and to control parasitemia. The results of these studies, which suggested that RC-12 had remarkable potential as a prophylactic and radical curative agent, attracted the attention of investigators at the National Institute of Allergy and Infectious Diseases who were concerned with assessments of the activities of potential antimalarial drugs via studies in human volunteers. Their interests in and requirements for undertaking work with RC-12 led to a second series of studies concerned with the subacute toxicity of RC-12 in rhesus monkeys. A brief report on the results of selected segments of both groups of studies was presented in 1966 (40).

MATERIALS AND METHODS

Only materials and methods common to assessments of both antimalarial activities and toxicity, or to the majority of experiments in either of these categories, will be referred to here. Procedures peculiar to individual experiments will be set forth, along with the design features and results of the studies that they served.

Test compounds, preparation for use, and method of administration. The lots of RC-12 used in these studies were prepared in the laboratories of Farbenfabriken Bayer AG, Wuppertal-Elberfeld, Federal Republic of Germany, and were made available to us through the efforts and courtesy of Karl Koenig, Advisor to the Medical Research Department of that company. The first preparation used in our studies was the acetate salt (87.7% RC-12 base content), referred to as RC-12 acetate. Because of its deliquescence, this salt was packaged in 5-ml vials as an aqueous solution containing 100 mg of RC-12 base per ml. Although this preparation presented no problems in our experimental studies, it was obviously ill suited to clinical application, which it was hoped would follow. A search for a salt that could be formulated in capsules or tablets led investigators in the laboratories of Farbenfabriken Bayer AG to the water-soluble, nonhygroscopic naphthalenedisulfonate (60% RC-12 base content), referred to as RC-12 NDS. Both this salt, provided as bulk powder, and the ampouled solution of RC-12 acetate were assessed for antimalarial activities and toxicity. Whatever their application, "stock" solutions of these salts containing from 10 to 50 mg of RC-12 base per ml, the concentration depending upon doses to be delivered, were prepared in distilled water immediately before administration.

The chloroquine diphosphate and primaquine diphosphate used in these studies were provided, respectively, as bulk powders by Sterling-Winthrop Research Institute, Rensselaer, N.Y., and Eli Lilly & Company, Indianapolis, Ind. Stock solutions of chloroquine, containing 5 mg of base per ml, and of primaquine, containing 1 mg of base per ml, were prepared in distilled water once weekly and stored at 4°C.

All compounds were administered by stomach tube once daily between 8:00 and 9:00 a.m., approximately 2 h after the morning feeding: in the case of monkeys used in the malaria studies, immediately after preparation of blood films; and in the case of monkeys used in the toxicity experiments, immediately after withdrawal of blood for hematological and biochemical studies. Whether committed to assessments of antimalarial activities or toxicity, monkeys were weighed on the day before the initial dose and on days 3, 5, 8, 10, 13, 15, etc., after the start of treatment. Doses for days 1 to 3 were based on the weights obtained before the start of the study. Doses for days 4 and 5 were based on the day 3 weights, doses for days 6 to 8 on day 5 weights, etc. Before each dosage was given, the volume(s) of stock solution(s) required for an individual monkey was pipetted into a 125-ml Erlenmeyer flask and diluted to 30 ml with chilled (4°C) distilled water. This dilute solution was transferred to a 50-ml beaker, drawn into a 30-ml glass syringe, and delivered via stomach tube to the appropriate recipient, to be followed by a 30-ml tap water rinse of flask, beaker, syringe, and stomach tube. Doses of all test compounds have been referred to as base throughout the Results and Discussion sections of this report.

Monkeys. A total of 306 juvenile, subadult, and young adult feral rhesus monkeys (*Macaca mulatta*), imported directly from New Delhi, India, were used in these studies: 217 for assessments of antimalarial activity and 89 for evaluations of toxicity. The assignees to each of these investigations included approximately equal numbers of males and females. At the time of assignment to an experiment, 94 of the 306 monkeys weighed between 2.3 and 2.9 kg, 147 weighed between 3.0 and 3.9 kg, 56 weighed between 4.0 and 4.9 kg, and 9 weighed between 5.2 and 7.4 kg. The procedures for acquiring these monkeys, transporting them from New Delhi to Davis, Calif., and conditioning them for experimental use, routine colony husbandry practices (including dietary management), and methods of handling for all facets of the studies without resort to sedatives, tranquilizers, anesthetics, or squeeze cages were identical with those described previously (33, 35, 37).

Assessments of prophylactic, radical curative, and suppressive activities. The *B* and *Ro/PM* (pyrimethamine-resistant) strains of *P. cynomolgi* were used in these studies. The latter strain served a single assessment of prophylactic activity, and the *B* strain served all other assessments of prophylactic activity and all assessments of radical curative and suppressive activities. The origins of these strains, their maintenance via serial monkey-to-mosquito-to-monkey passages, the characteristics of untreated sporozoite-induced and trophozoite-induced infections, and the responses of such infections to standard blood and tissue schizonticidal drugs have been detailed previously (35, 36, 39).

The evaluations of prophylactic and radical curative activities were carried out on monkeys inoculated intravenously with 7.9×10^4 to 1.2×10^6 sporozoites derived from the ground thoraces of *Anopheles freeborni* with heavily infected salivary glands. The procedures used to acquire lots of well-infected mosquitoes, monitor development of gut and salivary gland infections, harvest sporozoites from ground mosquito thoraces, and enumerate numbers of sporozoites in the inoculum were identical with those described elsewhere (38). Evaluations of suppressive activity were carried out on monkeys inoculated intravenously with 5×10^5 trophozoites derived from the blood of an untreated monkey in the serial monkey-to-mosquito-to-monkey passage line (35).

Assessments of prophylactic, radical curative, and suppressive activities rested on the results of repetitive searches of blood films for parasites. The procedures used in preparing thick and thin blood films, staining them with Giemsa, and detecting and quantifying parasitemias, as well as sched-

uling smear preparations, were identical with those detailed previously for comparable evaluations of other agents (36). The criteria employed in these studies for full protection against sporozoite challenge (prophylaxis), radical cure, and suppressive cure were identical with those described and validated elsewhere (36). In brief, the yardstick for prophylaxis was either full susceptibility to rechallenge with sporozoites, carried out 60 or more parasite-free days after the initial challenge, or absence of parasitemia for 76 or more days after challenge, including at least 21 days after splenectomy. The criterion for radical cure was the absence of parasitemia for 21 or more days after splenectomy which had been performed 60 or more thick-blood-film-negative days after delivery of the last dose of RC-12 or other agent to a monkey with an established sporozoite-induced infection. The yardstick for suppressive cure was the absence of parasitemia for a minimum of 60 days after administration of the last dose of test agent to a monkey with an established trophozoite-induced infection.

Assessments of toxicity. These evaluations included a very limited study of the acute oral toxicity of RC-12 and five relatively substantial studies of the subacute oral toxicity of this compound. In four of the five assessments, RC-12 was administered for 15 to 17 days; in one, it was administered for 225 days. The shorter studies dealt with the toxicity of RC-12 acetate administered alone, the impacts of concomitant administration of chloroquine on the toxicity of this salt, and the comparative toxicities of RC-12 acetate and RC-12 NDS. The longer study dealt only with the toxicity of RC-12 NDS.

In all of the subacute toxicity studies, monkeys were followed closely for evidences of impaired hematopoietic function. For 2 weeks before and throughout administration of RC-12, measurements of the numbers of erythrocytes, leukocytes, reticulocytes, and platelets, the distribution of various leukocytes, and the hematocrits and levels of hemoglobin in circulating blood were made at least twice weekly. Both the right and left femurs were removed at necropsy and split longitudinally. Specimens of marrow taken from the proximal, medial, and distal segments of the cavity were weighed and homogenized in 10-fold weights of pooled normal monkey serum. These homogenates were used to determine total numbers of cells per milligram of marrow and the distribution of cell types as visualized on Giemsa-stained smears.

Assessments of disturbances in renal and hepatic functions were limited to monkeys committed to the comparison of the toxicities of RC-12 acetate and RC-12 NDS and to the 225-day study of the latter salt. Measurements of the concentrations of urea (21) and glucose (10) in whole blood, serum glutamic oxalacetic transferase (SGOT) (42), glutamic pyruvic transferase (SGPT) (42), alkaline phosphatase (20), and prothrombin (26) activities, and the total bilirubin (19) content of serum were made twice weekly for a week before and throughout the period of dosage with RC-12, and in a few monkeys for 14 to 17 days thereafter.

Monkeys committed to the various toxicity studies were under continuous observation day and night for evidences of both acute and subacute reactions. Necropsies were started within 1 h of death of animals that succumbed to treatment or immediately after sacrifice. All monkeys that survived treatment with RC-12 in doses of 25.0 mg/kg or greater were sacrificed by overdosage with sodium pentobarbital and, like those that died, were subjected to a systematic necropsy. Animals were routinely sacrificed the day after the last dose of RC-12, except for a group of four kept under observation for 14 to 17 days posttreatment. Heart, liver, spleen, adrenals, and kidneys were removed first, blotted free of blood, and weighed. Specimens of these organs and of the other thoracic and abdominal viscera, the thyroids, and marrow from half of the slit femurs referred to above were fixed in Zenker containing 3% Formalin, processed for embedding in paraffin, and sectioned. Adjacent sections were stained with hematoxylin-eosin and Masson's trichrome. In addition, sections of femoral marrow were stained with Giemsa. Brain and spinal cord through the lumbar area were removed and fixed in 10% Formalin. Blocks of tissue from selected areas of the cerebral hemispheres and cerebellum, and the pons, medulla, and various levels of the spinal cord were processed for paraffin embedding and sectioned. Alternate sections in the ribbons from each block were mounted and stained with Morgan's iron-alum-hematoxylin-eosin and gallocyanin.

RESULTS

Antimalarial properties. (i) Prophylactic activity. The initial and major studies in this area dealt with the capacities of RC-12 acetate and RC-12 NDS to provide protection against sporozoite challenges when administered at 24-h intervals throughout the incubation period, specifically on the day before inoculation, 2 h before inoculation, and on days 1 through 7 thereafter. Five separate studies were included in this assessment. Four were concerned with the capacity of RC-12 to prevent infections with the *B* strain and one with the capacity of RC-12 acetate to prevent infections with the *Ro/PM* strain. The inocula for these experiments, in all cases in excess of 10^5 times the minimal infective dose, ranged from 6.4×10^5 to 1.2×10^6 sporozoites. Untreated control monkeys were included in each of the experiments. Primaquine, administered at a daily dose of 1.0 mg/kg, served as a positive drug control in two experiments with the *B* strain. Chloroquine, administered at a daily dose of 5.0 mg/kg, served as a negative drug control in a single experiment with the *B* strain. A total of 133 monkeys were committed to the five experiments.

The results of the four experiments with the *B* strain (Table 1) show that protection was complete when either salt of RC-12 was administered at doses of 25.0 or 100.0 mg/kg. At doses of 6.25 mg/kg, 9 of 10 recipients of RC-12 acetate and 15 of 21 recipients of RC-12 NDS were fully protected; the onset of parasitemia in one of the six treatment failures on RC-12 NDS was delayed for 49 days. All recipients of 1.56-mg/kg doses of either salt and of 0.39-mg/kg doses of the acetate developed patent infections, with at most a delay of 4 days in onset of parasitemia. In keeping with previous experience (34, 36), recipients of primaquine in a dose of 1.0 mg/kg were fully protected, whereas all recipients of chloroquine in doses of 5.0 mg/kg developed patent parasitemias.

The results of a more limited assessment of the capacity of RC-12 to prevent infections with the *Ro/PM* strain (Table 2) show that this agent, administered as the acetate, provided complete protection at doses of 25.0 or 100.0 mg/kg and protected two of three monkeys at a dose of 6.25 mg/kg. The third recipient of the latter dose became infected 7 days after onset of parasitemia in the untreated control. There is nothing in these data to suggest that the activity of RC-12 was either compromised or enhanced by pyrimethamine resistance.

The results of the major evaluation of the capacity of RC-12 to prevent infections with the *B* strain when administered in daily doses throughout the incubation period led to

TABLE 1. Capacity of RC-12 to prevent infections with sporozoites of the *B* strain of *P. cynomolgi*

Prophylactic regimen			Efficacy of regimen			
Compound	Daily dose[a]	No. of recipients[b]	No. of recipients fully protected	Recipients not fully protected: No.	Recipients not fully protected: Day of patency after challenge[c]	Recipients not fully protected: Days of delay in onset of patency[d]
Chloroquine	5.0	5	0	5	8 (4), 18	0 (4), 10
Primaquine	1.0	10	10	0		
RC-12	0.39[e]	5	0	5	8 (5)	0 (5)
	1.56[e]	5	0	5	8 (3), 9, 12	0 (3), 1, 4
	1.56[f]	4	0	4	9 (3), 11	1 (3), 3
	6.25[e]	10	9	1	10	2
	6.25[f]	21	15	6	9, 12 (3), 13, 57	1, 4 (3), 5, 49
	25.0[e]	15	15	0		
	25.0[f]	16	16	0		
	100.0[e]	5	5	0		
	100.0[f]	5	5	0		

[a] The dose (as milligrams of base equivalent per kilogram of body weight) indicated was administered once daily, the day before sporozoite challenge, 2 h before challenge, and for 7 consecutive days thereafter.

[b] The inocula for these recipients ranged from 6.4×10^5 to 1.2×10^6 sporozoites. Twenty-two untreated monkeys served as controls for these evaluations; prepatent periods were 8 days for 20 subjects and 10 and 11 days for the remaining 2 subjects.

[c] The numbers in parentheses refer to the numbers of recipients in whom parasitemias became patent on specific days.

[d] In calculating the number of days of delay in onset of patency, it was assumed that parasitemias of all untreated controls were patent on day 8. The numbers in parentheses indicate the numbers of recipients with specific days of delay in onset of patency.

[e] RC-12 was administered as the acetate.

[f] RC-12 was administered as the NDS.

two supplemental experiments. The first of these was aimed at determining whether a similar level of protection could be achieved with a once-weekly dosage regimen initiated at various times after sporozoite inoculation. RC-12 was administered as the acetate salt in a dose of 25.0 mg/kg. A total of 30 monkeys, six groups of five each, were committed to this experiment. Four groups received doses of RC-12 on days 0 and 7, 2 and 9, 4 and 11, or 6 and 13 after sporozoite challenge. A fifth group received this agent in the conventional 9-day regimen from the day before sporozoite challenge through day 7 thereafter. A sixth group served as untreated controls.

None of the five once-weeky regimens described above was as effective as the conventional 9-consecutive-day regimen in preventing infections (Table 3). The most effective of the divided dose regimens was that in which RC-12 was delivered 2 h before sporozoite challenge and on day 7 thereafter. This schedule provided full protection to two recipients and delayed the onset of patency by 7, 8, and 8 days in the remaining three. The least effective regimen was that in which the first dose of RC-12 was administered 6 days after challenge. Parasitemias of four of the recipients of this regimen became patent 8 days after inoculation, that of one on day 9, a result only slightly different from that in the untreated controls. Although dosage with RC-12 initiated 2 and 4 days after challenge did not fully protect any recipient, it did effect delays in onset of parasitemia by 4, 5, 8, 8, and 9 days in the five subjects in the day 2 group and by 2, 2, and 4 days in three members of the day 4 group.

The total dose of RC-12 in the divided dose arms of the

TABLE 2. Capacity of RC-12 to prevent infections with sporozoites of the *Ro/PM* strain of *P. cynomolgi*

Prophylactic regimen		Efficacy of regimen			
Daily dose[a]	No. of recipients[b]	No. of recipients fully protected	Recipients not fully protected: No.	Recipients not fully protected: Day of patency after challenge	Recipients not fully protected: Days of delay in onset of patency
6.25	3	2	1	15	7
25.0	3	3	0		
100.0	3	3	0		

[a] RC-12 was administered (as milligrams of base equivalent per kilogram of body weight) as the acetate, once daily, the day before sporozoite challenge, 2 h before challenge, and for 7 consecutive days thereafter.

[b] The inoculum for this experiment was 8.9×10^5 sporozoites. One untreated monkey served as control; prepatent period was 8 days.

TABLE 3. Capacity of once-weekly doses of RC-12 to prevent infections with sporozoites of the *B* strain of *P. cynomolgi*

Prophylactic regimen		Efficacy of regimen			
Days of dosage[a]	No. of recipients[b]	No. of recipients fully protected	Recipients not fully protected: No.	Recipients not fully protected: Day of patency after challenge[c]	Recipients not fully protected: Days of delay in onset of patency[d]
0 and 7	5	2	3	15, 16 (2)	7, 8 (2)
2 and 9	5	0	5	12, 13, 16 (2), 17	4, 5, 8 (2), 9
4 and 11	5	0	5	8 (2), 10 (2), 12	0 (2), 2 (2), 4
6 and 13	5	0	5	8 (4), 9	0 (4), 1
−1, 0, 1–7	5	5	0		

[a] RC-12 was administered as the acetate at a dose of 25.0 mg of base equivalent per kg body weight on the indicated days relative to the day of sporozoite challenge.

[b] The inoculum for this experiment was 6.4×10^5 sporozoites. Five untreated monkeys served as controls; prepatent periods were 8 days for all five subjects.

[c] The numbers in parentheses indicate the numbers of recipients in whom parasitemias became patent on specific days.

[d] The numbers in parentheses indicate the numbers of recipients with specific days of delay in onset of patency.

above assessment was 50.0 mg/kg; that in the consecutive dose arm was 225.0 mg/kg. This dosage differential might be viewed as prejudicing the performance of at least the most effective of the spaced regimens. This view is probably not supportable, for RC-12 administered as the acetate salt in a total dose of 56.25 mg/kg (6.25 mg/kg, daily for 9 days) provided full protection to 9 of 10 monkeys (Table 1).

The second of the supplemental studies was designed to determine for how long after the last of nine consecutive daily doses of RC-12 monkeys were protected against sporozoite challenge. This indirect assessment of the persistence of RC-12 was made necessary by the lack of a satisfactory procedure for measuring the concentrations of this agent and possibly its active metabolites in body fluids and tissues. A total of 24 monkeys, divided into six equally sized groups, were committed to this experiment. Included were a group of untreated controls and five groups of treated monkeys, the latter groups being dosed daily with RC-12 for 9 days on a staggered schedule such that all could be challenged at the same time but on either the day of the last dose or 2, 4, 6, or 8 days thereafter. Two monkeys in each group received doses of 12.5 mg/kg, and two received doses of 50.0 mg/kg administered as the NDS. The lower of these doses was selected because it was midway between the nearly fully effective and the fully effective doses of 6.25 and 25.0 mg/kg (Table 1). The larger dose was selected because it was double the fully effective dose.

The results of this experiment show that one of the two recipients of the 12.5-mg/kg dose, challenged on the last day of dosage, was fully protected; onset of patency was delayed but 3 days in the companion monkey. There was no evidence of persistence of protection in the monkeys challenged 2, 4, 6, or 8 days after the last of the 12.5-mg/kg doses (Table 4). In contrast, full protection persisted for 6 days after the last of the 50.0-mg/kg doses; even at 8 days, there were 6- and 7-day delays in onset of patency in the recipients of these doses (Table 4). These results suggest that persistence of RC-12 plays no significant role in the protection accorded by regularly effective doses. Whether the protracted protection accorded by a multiple of such doses is exploitable remains to be determined.

TABLE 4. Persistence of the capacity of RC-12 to prevent infections with sporozoites of the *B* strain of *P. cynomolgi* after completion of prophylactic regimen

Prophylactic regimen			Efficacy of regimen			
Days between last dose and sporozoite challenge	Daily dose[a]	No. of recipients[b]	No of recipients fully protected	Recipients not fully protected		
				No.	Day of patency after challenge[c]	Days of delay in onset of patency[d]
8	12.5	2	0	2	8 (2)	0 (2)
	50.0	2	0	2	14, 15	6, 7
6	12.5	1[e]	0	1	8	0
	50.0	2	2	0		
4	12.5	2	0	2	8 (2)	0 (2)
	50.0	2	2	0		
2	12.5	2	0	2	9 (2)	1 (2)
	50.0	2	2	0		
0	12.5	2	1	1	11	3
	50.0	2	2	0		

[a] RC-12 was administered (as milligrams of base equivalent per kilogram of body weight) as the NDS once daily for 9 consecutive days.

[b] The inoculum for this experiment was 7.9×10^4 sporozoites. Four untreated monkeys served as controls; prepatent periods were 8 days for three subjects and 9 days for one.

[c] The numbers in parentheses indicate the numbers of recipients in whom parasitemias became patent on specific days.

[d] In calculating the number of days of delay in the onset of patency, it was assumed that parasitemias of all untreated controls were patent on day 8. The numbers in parentheses indicate the numbers of recipients with specific days of delay in onset of patency.

[e] One of the monkeys in this subgroup developed dysentery 3 days after sporozoite challenge and was removed from the experiment.

TABLE 5. Capacity of RC-12 to cure established infections with sporozoites of the *B* strain of *P. cynomolgi*

Daily dose[a]	No. of infections treated	Primary response to treatment with RC-12				No. of recrudescences cured by follow-up chloroquine treatment[d]/ no. treated
		Day of parasite clearance[b]	No. of infections		Days from last dose to recrudescence	
			Cured	Recrudesced[c]		
6.25	3	6, 6, 7	0	3	7, 9, 13	0/1[e]
25.0	9	5 (8), 6	3	6	12, 13, 14, 14, 16,17	4/6
100.0	6	5, 6 (5)	2	4	6, 7, 22, 26	4/4

[a] RC-12 was administered (as milligrams of base equivalent per kilogram of body weight) as the acetate once daily for 7 consecutive days.

[b] The numbers refer to day after first dose of RC-12. The numbers in parentheses indicate the numbers of recipients with clearance of parasitemia on the specified day.

[c] Reappearance of parasitemia was termed recrudescence because of the high rate of cures after treatment with chloroquine.

[d] Chloroquine was administered in a dose of 2.5 mg of base per kg body weight once daily for 7 consecutive days.

[e] Two infections that recrudesced after dosage with RC-12 alone were retreated with a combination of RC-12 and chloroquine.

(ii) Radical curative activity. The capacity of RC-12 to cure established infections with sporozoites of the *B* strain was evaluated in three separate studies. The first was concerned with the activity of the acetate salt delivered in a monodrug regimen. The 18 monkeys committed to this experiment were derived from the first two assessments of prophylactic activity and included three that received chloroquine, four that received 0.39 mg of RC-12 per kg, four that received 1.56 mg of RC-12 per kg, one that received 6.25 mg of RC-12 per kg, and six of the untreated controls. These 18 monkeys were divided into subgroups of three, nine, and six and treated with RC-12 in doses of 6.25, 25.0, or 100.0 mg/kg once daily for 7 consecutive days. Treatment was started in the ascending phase of the primary attack, when parasitemias involved 10 to 40 of each 10^4 erythrocytes.

Clearance of parasitemia was attained in all 18 subjects within 5 to 7 days of the initial dose of RC-12 (Table 5). Infections in three of the nine recipients of doses of 25.0 mg/kg and in two of the six recipients of doses of 100.0 mg/kg were cured. Parasitemias of the remaining 10 recipients of these doses and of the 3 recipients of 6.25 mg/kg were again patent 6 to 26 days after the last dose. Within 3 days of patency, the recurring infections of 11 of the 13 monkeys were treated with chloroquine at a dose of 2.5 mg/kg once daily for 7 consecutive days. This treatment course effected cure of 8 of the 11 infections, 4 originally treated with RC-12 at doses of 25.0 mg/kg and 4 at doses of 100.0 mg/kg. This response indicated that the reestablishment of parasitemia in at least these eight subjects stemmed from persisting blood

TABLE 6. Capacity of RC-12 to cure established infections with trophozoites of the *B* strain of *P. cynomolgi*

Daily dose[a]	Application of regimen		Response to treatment				
	No.	Treatment course	Days from initial dose to first of series of negative thick blood films	No. of cures	No. of persisting parasitemias	Clearance of parasitemia followed by recrudescence	
						No.	Days from last dose to recrudescence
1.56	3	Initial	RNC[b], RNC, RNC	0	3	0	
6.25	3	Initial	6, 7, RNC	0	1	2	4, 8
	2	1st retreatment	6, 7	0	0	2	9, 14
25.0	3	Initial	6, 6, 6	0	0	3	6, 9, 11
	3	1st retreatment	4, 5, 6	1	0	2	12, 18
	2	2nd retreatment	2, 4	2	0	0	
100.0	3	Initial	6, 6, 6	0	0	3	13, 15, 18
	3	1st retreatment	3, 6, 6	0	0	3	21, 23, 23
	2	2nd retreatment	4, 6	0	0	2	23, 24

[a] RC-12 was administered (as milligrams of base equivalent per kilogram body weight) as the acetate once daily for 7 consecutive days.
[b] RNC, Parasitemia was reduced but not to level of negativity on thick blood films.

schizonts and that altogether the initial treatment with RC-12 had eradicated the tissue schizonts (the essential requirement for radical cure) in seven of the nine recipients of 25.0-mg/kg doses and in six of six recipients of 100.0-mg/kg doses.

The results of this experiment made it necessary to assess the activity of RC-12 against infections with trophozoites. This assessment, although not in the radical cure category, is described here because it produced results that determined the design of subsequent appraisals of radical curative activity. The experiment involved work with a group of 13 monkeys, each inoculated with 5×10^5 trophozoites of the *B* strain; 1 served as an untreated control and the remaining 12, in subgroups of 3, received RC-12 in doses of 1.56, 6.25, 25.0, and 100.0 mg/kg once daily for 7 consecutive days. Treatment with these doses was initiated in the ascending phase of the primary attack when 10 to 40 of each 10^4 erythrocytes were parasitized. When parasitemias persisted or reappeared after apparent clearance, second and, if required, third courses at higher doses were applied, except for infections originally treated with 100.0 mg of RC-12 per kg, concerns with toxicity precluding dosage above that level.

The results of this experiment, covering a total of 24 treatment courses, showed that doses of RC-12 ranging from 6.25 to 100.0 mg/kg regularly reduced parasitemias to the thick-blood-film-negative level. In most subjects, this reduction took place within 6 days of the first dose, but with three exceptions, one in a first retreatment course and two in a second, doses of RC-12 failed to eradicate all parasites (Table 6). Doses of 1.56 mg/kg suppressed parasitemia significantly but did not effect clearance. These results, showing that RC-12 had significant activity against blood schizonts but could not be depended upon for eradication of these forms, led to the concomitant administration of chloroquine in all subsequent assessments of the radical curative activity of this pyrocatechol.

The second major study of the curative activity of RC-12 was concerned with the capacity of this agent to eradicate established sporozoite-induced infections when administered as either the acetate or the NDS salt concomitantly with chloroquine. The 57 monkeys committed to this evaluation included 43 with previously untreated infections; 18 were inoculated for assessment of the activity of the acetate salt, and 25 were inoculated for a comparison of the activities of the acetate and NDS salts. Of the remaining 14 monkeys, 8 were derived from assessments of the prophylactic activity of RC-12 and had been treated with either this agent or chloroquine during the primary attack or first relapse. The remaining six monkeys had served an assessment of the prophylactic and curative activity of the 6-aminoquinoline B-505, with entirely negative results (31). In the test for cure, RC-12 was administered once daily for 14 days in doses ranging from 0.39 to 100.0 mg/kg concomitantly with chloroquine in daily doses of 2.5 mg/kg for the first 7 days. The 14-day schedule for RC-12 was the counterpart of the conventional primaquine treatment regimen. The 7-day schedule for chloroquine was one that has been shown to be completely effective in eradicating infections with trophozoites of the *B* strain (36). Treatment of previously untreated infections was initiated early in the primary attack, when parasitemias involved from 10 to 40 of each 10^4 erythrocytes. Treatment of relapses, whether in the special group of monkeys or in those receiving RC-12 in the primary attack, was initiated within 3 days of reestablishment of patency.

At daily doses of 0.39 and 1.56 mg/kg, RC-12 showed no evidence of curative activity (Table 7). In contrast, delivery of doses of 6.25 and 25.0 mg/kg effected cure of 22 of 32 (69%) and 28 of 30 (93%) infections, respectively. It is noteworthy that, when these doses did not effect cure, relapse was markedly delayed, for 64 and 96 days in 2 of the 10 treatment failures at doses of 6.25 mg/kg and for 82 and 85 days in the 2 treatment failures at doses of 25.0 mg/kg. In three of these four cases, relapse did not occur until 10 to 21 days after splenectomy, performed as the ultimate test of cure. Such extensions of the relapse interval reflect an extremely small residue of tissue shizonts and indicate that RC-12 at a dose of 6.25 mg/kg came very close to curing 24 of 32 infections and, at a dose of 25.0 mg/kg, 30 of 30 infections.

Although the assessment of the comparative curative activities of RC-12 acetate and RC-12 NDS was not only limited in dimensions but skewed with respect to distribution of numbers of recipients, the available data indicate that the accomplishments of these salts were very similar, if not

TABLE 7. Capacity of RC-12, administered in combination with chloroquine, to cure established infections with sporozoites of the *B* strain of *P. cynomolgi*

Daily dose of RC-12[a]	Application of regimen		Response to treatment		
			No. of infections		Days from last dose of chloroquine to relapse
	No.	Attack treated[b]	Cured	Relapsed	
0.39[c]	3	P	0	3	6, 6, 8
1.56[c]	3	P	0	3	6, 8, 12
6.25[c]	12	P	7	5	27, 28, 30, 38, 96[d]
6.25[c]	14	R1	10	4	20, 24, 30, 31
6.25[c]	2	R2	2	0	
6.25[e]	4	P	3	1	64
25.0[c]	11	P	11	0	
25.0[c]	6	R1	6	0	
25.0[c]	4	R2	3	1	82[f]
25.0[c]	2	R3	1	1	85[g]
25.0[e]	6	P	6	0	
25.0[e]	1	R1	1	0	
100.0[e]	4	P	4	0	

[a] RC-12 was administered (as milligrams of base equivalent per kilogram of body weight) in the dose indicated once daily for 14 consecutive days; chloroquine, in a daily dose of 2.5 mg of base equivalent per kg of body weight, was administered concomitantly for the first 7 of these days.
[b] P, Primary attack; R1, R2, and R3, first, second, and third relapses, respectively.
[c] RC-12 was administered as the acetate.
[d] This relapse occurred 21 days after splenectomy.
[e] RC-12 was administered as the NDS.
[f] This relapse occurred 10 days after splenectomy.
[g] This relapse occurred 15 days after splenectomy.

identical. Thus, at a dose of 6.25 mg/kg, 19 of 28 recipients of the acetate (68%) and 3 of 4 recipients of the NDS (75%) were cured. At a dose of 25.0 mg/kg, 21 of 23 recipients of the acetate (91%) and 7 of 7 recipients of the NDS (100%) were cured.

The third major study was concerned with the impact of the duration of treatment on the capacity of RC-12 to eradicate established infections with sporozoites of the *B* strain. This issue was pursued in two separate experiments of identical design concerned, respectively, with the activities of the acetate and NDS salts. The inocula for these experiments were 6.4×10^5 and 2.8×10^5 sporozoites. A group of 21 monkeys was committed to the first experiment, a group of 23 to the second. RC-12 was administered in a daily dose of 25.0 mg/kg in both experiments: for 4, 7, and 14 consecutive days in the first and for 2, 4, 7, and 14 days in the second. Irrespective of the duration of dosage with RC-12, chloroquine was administered in a dose of 5.0 mg/kg once daily for 4 consecutive days. Previous studies have shown that this regimen and that of 2.5 mg/kg for 7 days are equally effective in eliminating the erythrocytic phase of the infection (29, 36). Treatment was initiated during the ascending phase of the primary attack, when 10 to 40 of each 10^4 erythrocytes were parasitized and within 3 days of reestablishment of patency in relapses.

None of the six infections treated with RC-12 NDS for 2 days was cured (Table 8); however, the intervals to relapse were longer, by 5 to 16 days, than those associated with delivery of chloroquine alone (29, 30, 36). Treatment for 4 days effected cure of 11 of the 19 infections. The relapses of six of the eight infections not cured were delayed for more than 30 days. All 20 infections treated with RC-12 for 7 days and all 12 treated for 14 days were cured. Although data on this issue are extremely limited, there appeared to be no marked difference in the curative activities of RC-12 acetate and RC-12 NDS. The results of this study suggest that delivery of RC-12 for 7 days will be required for cure, but this suggestion is tempered by recognition that the total doses delivered in the 4- and 7-day regimens were different. The accomplishments in the 4-day regimen, at only 57% of the total dose applied in the 7-day regimen, would suggest that overall performance of these regimens might have been the same had the total doses delivered been identical.

Toxicological Characteristics. (i) Acute toxicity. Four monkeys, discarded as cured from an unrelated malaria therapy study, served a single preliminary assessment. Two received RC-12 acetate in single oral doses of 100.0 mg/kg; two received single doses of 500.0 mg/kg. There were neither immediate reactions to the lower of these doses nor delayed reactions over a 5-day post-dose observation period. Both recipients of the larger dose exhibited severe clonic convulsions within 30 min of dosage. One died 20 min after onset of these seizures. The convulsions in the second monkey abated within 10 min but left this subject weak and severely depressed for approximately 24 h. Thereafter, his behavior in his cage and upon release into the cage room was entirely normal. Limited as they were, these results guided the selection of doses for both the initial appraisals of prophylactic and radical curative activities and the first of the repetitive-dose toxicity evaluations.

(ii) Subacute toxicity. The untoward reactions evoked by repeated daily doses of RC-12 were studied in five separate experiments, four of common design. The first two of these four, carried out with RC-12 acetate, were designed: (i) to identify the types of reactions evoked by RC-12 and the dose level at which these reactions occurred; (ii) to determine whether the concomitant administration of chloroquine modified the toxicity of RC-12 either qualitatively or quantitatively; and (iii) to provide the basis for developing a crude therapeutic index. These experiments, each lasting 15 days, were served by 25 monkeys; 14 received RC-12 alone, 8 received RC-12 in combination with chloroquine, and 3

TABLE 8. Influence of the duration of treatment with RC-12 on the capacity of this agent (administered in combination with chloroquine) to cure established infections with sporozoites of the *B* strain of *P. cynomolgi*

Days of treatment with RC-12[a] (total dose)	Applications of regimen		Response to treatment		
			No. of infections		Days from last dose of chloroquine to relapse
	No.	Attack treated[b]	Cured	Relapsed	
2 (50)	6[c]	P	0	6	15, 20, 24, 24, 26, 31
4 (100)	7[d]	P	5	2	12, 36
	6[c]	P	3	3	12, 45, 73
	6[c]	R1	3	3	34, 43, 49
7 (175)	7[d]	P	7	0	
	2[d]	R1	2	0	
	6[c]	P	6	0	
	3[c]	R1	3	0	
	2[c]	R2	2	0	
14 (350)	7[d]	P	7	0	
	5[c]	P	5	0	

[a] RC-12 was administered in a dose of 25.0 mg of base equivalent per kg of body weight once daily for 2, 4, 7, or 14 days and with chloroquine in a dose of 5.0 mg of base per kg of body weight for 4 days.
[b] P, Primary attack; R1 and R2, first and second relapses, respectively.
[c] RC-12 was administered as the NDS.
[d] RC-12 was administered as the acetate.

served as untreated controls. The third experiment, also lasting 15 days, was designed to compare the toxicities of RC-12 acetate and RC-12 NDS. It was served by 20 monkeys; 9 received RC-12 acetate, 9 received RC-12 NDS, and 2 served as untreated controls. The fourth experiment, lasting 17 days, was aimed at expanding the data on the toxicity of RC-12 NDS acquired in the third experiment and was served by 18 recipients of the test agent and 2 untreated controls. Reference should be made to the Materials and Methods section for details of experimental procedures common to these four experiments.

The fifth experiment was designed to determine whether long-term administration of RC-12 NDS, at the daily doses required for prophylaxis and radical cure and twice the latter level (i.e., at 12.5, 25.0, and 50.0 mg/kg), would evoke evidences of hepatotoxicity or ocular toxicity. Concerns with the toxicity of RC-12 for the liver stemmed from the results of the third and fourth, 15- to 17-day subacute studies, as described below. Concerns with the toxicity of RC-12 for the eye and associated structures stemmed from a report of an investigation of the toxicity of RC-12 for beagle dogs, carried out in the Huntingdon Research Centre, Huntingdon, England, under contract with Farbenfabriken Bayer AG. This report (personal communication, D. Lorke, Director, Institut für Toxikologie, Farbenfabriken Bayer AG, 30 March 1967), showed that there was a loss of the usual green color and deposition of fine black pigment in the tapetum lucidum of six of six dogs given daily doses of 80.0 mg of RC-12 per kg for 11 weeks and one of six dogs given doses of 40.0 mg/kg for that period. Although the rhesus monkey does not have a tapetum lucidum, earlier experiences on the ocular toxicity of ethambutol (28) suggested that the observations on the canine eye should not be ignored and that the impacts of RC-12 on structure and function of the simian eye deserved attention.

The resulting long-term study, originally planned as a 180-day experiment, was extended to 225 days because of the absence of key personnel on the scheduled termination date. It was served by 20 monkeys; 5 served as untreated controls and three groups of 5 were given RC-12 in daily doses of 12.5, 25.0, and 50.0 mg/kg. Reference should be made to the Materials and Methods section for all experimental procedures employed in this experiment, except those pertinent to disturbances in visual function. The latter were performed on both treated and control monkeys twice before the start of the experiment and once every 2 weeks thereafter. At these times, animals were subjected to a careful ophthalmoscopic examination while being gently restrained in a sitting position by an animal caretaker. The monkeys were then placed in a restraining chair with head held firmly but with arms free to push away a 2-mm-diameter dowel stick as it was moved toward the orbit from various angles. After this test, the monkey was set free in a caged area (14 by 22 feet) with a number of randomly placed obstacles on the floor and pursued therein by an animal caretaker to determine whether capacity to avoid running into fixed objects was impaired. When animals were sacrificed at the end of the experiment, their eyes and approximately 15-mm lengths of the optic nerve attached thereto were removed, fixed in 10% Formalin, and processed for histopathologic study.

Reactions to both short- and long-term dosage with RC-12 alone and to short-term dosage of this agent in combination with chloroquine are summarized in Table 9. As indicated therein, there were no outward manifestations of toxicity in the recipients of RC-12 alone in doses of 3.125, 6.25, 12.5, 25.0, or 50.0 mg/kg daily for 15 or 17 days or in doses of 12.5, 25.0, or 50.0 mg/kg daily for 225 days, or to a 50.0-mg/kg dose of RC-12 with a 2.5-mg/kg dose of chloroquine for 15 days. Reactions to a dose of 100.0 mg/kg varied markedly. There were no outward expressions of toxicity in 13 of the 17 recipients of such doses daily for 15 days. The remaining four recipients suffered fatal reactions. One monkey began to convulse 35 min after the initial dose; these convulsions, clonic in character, increased in intensity until death occurred 25 min later. The other three monkeys exhibited no outward manifestations of toxicity for 3, 5, and 9 days but became severely depressed after doses 4, 6, and 10, exhibited progressive malaise and muscular weakness thereafter, and died or were sacrificed in extremis on days 9, 13, and 14 after the initial dose. A dose of 200.0 mg/kg was fatal to all seven recipients. Although all of these monkeys exhibited convulsive seizures at some time during the treatment course, the duration of dosage before the first appearance of seizures varied markedly. Two monkeys began to convulse 30 min after the initial dose and were dead 15 min later. In the remaining five monkeys, convulsions first appeared after 6 to 11 doses; these seizures, lasting no more than 5 min, were followed by profound malaise and muscular weakness and death on days 8 to 12 after the initial dose.

No dose of RC-12, whether tolerated for up to 225 days or lethal, evoked changes in the formed elements of peripheral blood and bone marrow, the concentrations of glucose or urea in whole blood, alkaline phosphatase activity or bilirubin level in serum, or prothrombin time. Sublethal doses of RC-12 had no effect on body weight. Recipients of lethal doses who survived for 8 or more days suffered 5 to 9% weight losses. Doses of RC-12 ranging from 12.5 to 200.0 mg/kg were without effect on the absolute and relative (to original body weight) weights of heart, spleen, adrenals, and kidneys. Doses of 12.5, 25.0, and 50.0 mg/kg administered daily for 225 days evoked no changes in the fundus of the eye detectable by ophthalmoscopic examination or objective evidences of loss of viscual acuity.

At doses of 50.0 mg/kg and greater, RC-12 effected both absolute and relative increases in liver size (Table 10). These increases were related to dose but not to the duration of dosage and were of the same order, regardless of whether RC-12 was administered as the acetate or as the NDS. The increases evoked by 50.0 mg of RC-12 per kg were neither enhanced nor diminished by concomitant dosage with chloroquine.

As indicated before, measurements of SGOT and SGPT activities were limited to the last three of the five assessments of the toxicity of RC-12. The data acquired in these appraisals showed that RC-12 in daily doses of 3.125, 6.25, 12.5, or 25.0 mg/kg for 17 to 225 days had no effect on SGOT activity but that, in daily doses of 50.0 mg/kg or greater, it produced dose-related increases in the activity of this enzyme (Table 11, groups 1 through 5). The impacts of various doses of RC-12 on SGPT activity paralleled those on SGOT activity. For this reason, changes in SGPT activity are not detailed here.

The mean SGOT activities of recipients of doses of 50.0, 100.0, and 200.0 mg/kg were characterized by sizable standard deviations (Table 11), implying considerable animal-to-animal variability. As indicated in the lower half of that table, the recipients of doses of both 50.0 and 100.0 mg/kg fell into two distinct families with respect to alterations in SGOT activity. Thus, in 8 of the 12 recipients of a dose of 50.0 mg/kg (group 3A), SGOT activities were maintained close to or at pretreatment levels throughout dosage; in the

TABLE 9. Reactions of rhesus monkeys to short- and long-term administration of RC-12

Dosage regimen			No. of monkeys		Reactions to administration of RC-12[d]
Daily dose of RC-12[a]	Salt of RC-12	Scheduled days of dosage[b]	Survivors of scheduled dosage/total	Deaths from dosage[c]	
0		15 (5), 17 (2)	7/7	0	None
0		225	5/5	0	None
3.125	NDS	17	3/3	0	No outward reactions; not sacrificed
6.25	NDS	17	3/3	0	No outward reactions; not sacrificed
12.5	NDS	17	3/3	0	No outward reactions; not sacrificed
12.5	NDS	225	5/5	0	None
25.0	Acetate	15	2/2	0	None
25.0	NDS	17	3/3	0	None
25.0	NDS	225	5/5	0	None
50.0	Acetate	15	7/7	0	No outward reactions; transient increases in SGOT and SGPT (3) levels; hepatomegaly
50.0	Acetate[e]	15	4/4	0	No outward reactions; hepatomegaly
50.0	NDS	15 (3), 17 (6)	9/9	0	No outward reactions; transient increases in SGOT and SGPT (6) levels; hepatomegaly
50.0	NDS	225	5/5	0	No outward reactions; transient increases in SGOT and SGPT (4) levels; hepatomegaly
100.0	Acetate	15	7/9	2 (1, 12)	One monkey exhibited convulsions 35 min after dose 1 and died 25 min later; a second monkey exhibited malaise and muscular weakness 120 min after dose 4, increasing in intensity with continued dosage, prostrated after dose 12, sacrificed before dosage on day 13. No outward reactions in the seven survivors. Progressive increases in SGOT and SGPT levels and hepatomegaly in all monkeys except the subject that died after initial dose
100.0	Acetate[e]	15	4/4	0	No outward reactions; hepatomegaly
100.0	NDS	15	2/4	2 (9, 14)	One monkey exhibited malaise after dose 6, a second after dose 10, progressing to prostration and death on days 9 and 14 after dosage on those days. No untoward reactions in the two survivors. Progressive increases in SGOT and SGPT levels and hepatomegaly were common to all subjects.
200.0	Acetate	15	0/5	5 (1, 1, 8, 9, 11)	Two monkeys exhibited convulsions approximately 30 min after dose 1 and died approximately 15 min later. Three monkeys exhibited much less severe convulsions after each of 8, 9, and 11 doses, followed by extreme malaise, prostration, and death on days 8, 9, and 11 after dosage on these days. Progressive increases in SGOT and SGPT levels and hepatomegaly were common to the latter two subjects; levels of SGOT and SGPT were not measured in the third subject
200.0	NDS	15	0/2	2 (11, 11)	Both monkeys exhibited convulsions approximately 30 min after doses 6 and 8, followed by extreme malaise, prostration after dose 10, and death on days 11 and 12 after 11 doses. Both exhibited progressive increases in SGOT and SGPT levels and hepatomegaly

[a] RC-12 was administered as milligrams of base equivalent per kilogram of body weight.
[b] Numbers in parentheses indicate the numbers of recipients of scheduled doses.
[c] Numbers in parentheses indicate the numbers of doses before death.
[d] Numbers in parentheses indicate the numbers of monkeys within dosage groups with elevations in levels of SGOT and SGPT.
[e] Monkeys in this group received concomitant daily doses of chloroquine (2.5 mg/kg).

TABLE 10. Liver weights of recipients of RC-12

Dosage regimen			Weight of liver as % of body weight (mean ± SD)[c]
Daily dose of RC-12[a]	Salt of RC-12	Scheduled days of dosage[b]	
0		15 (5), 17 (2)	2.78 ± 0.22
0		225	3.02 ± 0.27
25.0	Acetate	15	2.97 (2.78, 3.06)[d]
25.0	NDS	17	3.2 ± 0.21
25.0	NDS	225	3.07 ± 0.23
50.0	Acetate	15	3.67 ± 0.16
50.0	NDS	15 (3), 17 (6)	3.44 ± 0.83
50.0	NDS	225	3.62 ± 0.41
100.0	Acetate	15	4.73 ± 0.93
100.0	NDS	15	4.55 ± 0.68
200.0	Acetate	15	5.53 ± 0.19
200.0	NDS	15	6.43 (6.21, 6.66)[d]
50.0[e]	Acetate	15	3.26 ± 0.23
100.0[e]	Acetate	15	4.04 ± 0.25

[a] RC-12 was administered as milligrams of base equivalent per kilogram of body weight.
[b] Numbers in parentheses indicate numbers of recipients of scheduled doses.
[c] See totals in column 4, Table 9, for numbers of subjects contributing data.
[d] Liver weights of individual monkeys.
[e] Four monkeys in each of these regimens received 2.5 mg of chloroquine base equivalent per kg of body weight concomitantly with RC-12 as the acetate.

remaining four monkeys (group 3B), there were moderate but unequivocal elevations in SGOT activity. Similarly, in three of the eight recipients of a dose of 100.0 mg/kg (group 4A), elevations in SGOT activity were moderate; in the remaining five, they were very substantial.

Persistence of elevated SGOT activity posttreatment and the possibility that such activity might become elevated in the immediate post-dosage period, when it had not been elevated before, were studied in four recipients of a dose of 50.0 mg of RC-12 NDS per kg. In two of the four monkeys, there had been no change in SGOT activity during dosage; in two, SGOT activity had increased moderately (Table 12). SGOT activity did not change posttreatment in either member of the former group. In both members of the latter group, activity increased substantially during the first 2 to 4 post-treatment days and then declined, reaching pretreatment levels by post-dosage day 12.

Of the organs and tissues subjected to gross and microscopic study, only the liver exhibited departures from normal that could be attributed to RC-12. The departures in this organ included an increase in size (and weight), rounding of its usually sharp edges, and a shift in color from the characteristic deep red-maroon to deep brown. With respect to both incidence and intensity, these changes were related to dose size but not to dosage duration. They were not exhibited by recipients of a dose of 12.5 or 25.0 mg/kg but were present in 12 of 20 recipients of a dose of 50.0 mg/kg for 15 to 17 days, in 2 of 5 recipients of this dose for 225 days, and in all recipients of eight or more daily doses of 100.0 or 200.0 mg/kg.

There were no alterations in structure, demonstrable by light microscopy, in the liver of any recipient of a dose of 12.5 or 25.0 mg/kg. Changes in hepatocyte structure were present in all recipients of RC-12 in repeated doses of 50.0 mg/kg or greater. Vacuolation (possibly fatty infiltration) of these cells was prominent. This change was accompanied by a slight increase in cell size. With respect to incidence, numbers, and size of vacuoles, the intensity of this reaction varied directly with dose. At a dose of 200.0 mg/kg, essentially all cells in a section were involved. At a dose of 50.0 mg/kg, there was focal involvement. No more than 10 to 20% of the hepatocytes in a focus were affected. The degree of vacuolation in individual cells in these foci was clearly less than that found when nearly the total hepatocyte population was involved. Necrosis of hepatocytes and proliferative activity of Kupfer cells were not encountered. Apart from small clusters of lymphocytes, such as those found frequently in the livers of apparently healthy feral rhesus monkeys, there was no cellular reaction.

DISCUSSION

The studies described in this report have provided a relatively broad appraisal of the potentials of RC-12 as an antimalarial drug. Included were characterizations of (i) the capacities of this pyrocatechol derivative to control diverse phases of infections with *P. cynomolgi* in rhesus monkeys and (ii) its adverse effects on noninfected, otherwise normal monkeys.

TABLE 11. Effects of RC-12 on SGOT activity

Group	Daily dose of RC-12[a]	No. recipients of RC-12 as:		Mean ± SD activity of SGOT (U/ml) on following day of dosage:				
		Acetate	NDS	Pre	4	8	11	15
1	0	0	0	28.9 ± 9.3	25.5 ± 5.3	25.6 ± 2.5	20.0 ± 1.0	24.3 ± 5.0
2	3.125–25.0	0	12[b]	28.3 ± 2.9	26.1 ± 4.6	26.6 ± 6.2	20.3 ± 5.2	22.8 ± 5.1
3	50.0	4	8	27.3 ± 5.1	28.3 ± 3.3	35.6 ± 13.5	33.1 ± 12.4	43.4 ± 21.2
4	100.0	4	4	29.1 ± 6.0	37.4 ± 12.3	64.0 ± 20.0	129 ± 116	166 ± 160
5	200.0	2	2	24.2 ± 2.2	80.4 ± 39.9	100 ± 34.6	264 ± 126	
3A[c]	50.0	2	6	27.3 ± 4.5	29.1 ± 2.9	33.4 ± 5.9	29.7 ± 4.6	31.3 ± 7.1
3B[d]	50.0	1	3	27.4 ± 6.9	26.8 ± 3.9	40.1 ± 23.5	40.0 ± 20.5	67.8 ± 18.3
4A[e]	100.0	1	2	24.6 ± 5.3	27.7 ± 4.2	54.0 ± 20.4	59.8 ± 18.5	53.9 ± 17.8
4B[f]	100.0	3	2	31.7 ± 5.1	43.1 ± 12.1	69.9 ± 19.3	170 ± 132	241 ± 174

[a] RC-12 was administered as milligrams of base equivalent per kilogram of body weight.
[b] This group included four subgroups, each of three monkeys, recipients of daily doses of 3.125, 6.25, 12.5, or 25.0 mg base/kg body weight.
[c] This group included subjects from group 3 in whom SGOT activity during treatment exceeded activity pretreatment by <50%.
[d] This group included subjects from group 3 in whom SGOT activity during treatment exceeded activity pretreatment by >50%.
[e] This group included subjects from group 4 in whom SGOT activity during treatment exceeded activity pretreatment by less than fourfold.
[f] This group included subjects from group 4 in whom SGOT activity during treatment exceeded activity pretreatment by greater than fourfold.

TABLE 12. Sequence of changes in SGOT activity posttreatment

Monkey no.[a]	Activity of SGOT (U/ml) on:										
	Pretreatment[b]	Day of treatment				Day posttreatment						
		4	8	11	15	2	4	6	8	10	12	14
1723	30	28	27	28	29	37	26	33	26	28	25	22
1809	29	30	34	27	27	25	26	28	24	22	25	27
1726	30	27	26	28	56	147	69	36	31	26	26	26
1861	21	22	27	32	65	91	112	—[c]	64	—	32	30

[a] Each of these monkeys received RC-12 NDS in doses equivalent to 50.0 mg of base per kg body weight once daily for 17 days.
[b] These values are the means of four measurements carried out during a 14-day pretreatment interval.
[c] Hemolysis of blood sample on this day invalidated measurement of SGOT activity.

Characterization of the activity of RC-12 against the early tissue forms of *P. cynomolgi* showed that: (i) when this agent was delivered daily throughout the incubation period in doses of 6.25 to 25.0 mg/kg it provided either nearly complete or complete protection against challenges with 10^5 to 10^6 times the minimum infective dose of sporozoites; (ii) this protective capacity was not impaired by pyrimethamine resistance; (iii) protection accorded by a fully effective regimen of 12.5 mg/kg daily for 9 days did not persist beyond the last dosage day but could be extended by 6 days when the size of the daily dose was increased fourfold; and (iv) once-weekly doses provided protection only when delivered on the day of sporozoite challenge and even then was complete in only 40% of the applications.

Characterization of the activity of RC-12 against the developed and persisting tissue forms of *P. cynomolgi* showed that: (i) these forms could be eradicated, resulting in radical cure, by essentially the same daily doses required for protection against infections with sporozoites; (ii) because of the limited activity of RC-12 against the erythrocytic stages of the parasite, cure of established infections required concomitant dosage with an effective blood schizonticide, such as chloroquine; and (iii) the developed and persisting tissue forms could be eradicated by a dosage regimen as brief as 4 days.

Characterization of the activity of RC-12 against the erythrocytic stage of infections, although limited in scope, showed that: (i) parasitemia could be reduced to thick-blood-film-negative levels by daily doses of 6.25 mg/kg, but that the blood schizonts could not be eliminated completely by doses as large as 100.0 mg/kg; (ii) the time from first dose of RC-12 to reduction of parasitemia to thick-blood-film-negative status was independent of the size of the dose, within the above range; and (iii) the times required for such reduction in recrudescences were either the same or slightly shorter than those required on initial treatment, suggesting that parasites responsible for recrudescences were not resistant to RC-12.

Characterization of the subacute toxicity of RC-12 for the rhesus monkey showed that: (i) daily doses of 50.0 mg/kg or lower for 15 to 225 days evoked no outward manifestations of toxicity; (ii) doses of 100.0 and 200.0 mg/kg evoked fatal reactions referrable to central nervous system stimulation and depression; (iii) time between delivery of the first of the above doses and occurrence of fatal reactions was highly variable, from 45 min after the initial dose to 23 h after dose 11 in the recipients of 200.0 mg/kg; (iv) doses of 25.0 mg/kg and less for 15 to 225 days produced no discrete toxic reactions; (v) repetitive doses of 50.0 to 100.0 mg/kg produced hepatomegaly, elevations in the activities of transaminases in serum, and vacuolation of hepatocytes; (vi) the intensity of these manifestations of hepatotoxicity was dose related; (vii) the elevations in transaminase activities in recipients of a dose of 50.0 mg/kg did not increase with protracted dosage and were promptly reversible upon termination of the treatment; and (viii) concomitant administration of chloroquine did not affect the incidence or intensity of either overt or discrete manifestations of RC-12 toxicity.

Studies on the absorption, elimination, tissue disposition, and metabolic fate of RC-12 were included in the original plans for assessing the potential of this agent as an antimalarial drug. Their omission from this report, a significant shortcoming, stems from our inability to develop an acceptable analytical procedure during the 3-year period when RC-12 was under study. Efforts to apply methyl orange coupling (3) and induced fluorescence (4, 8, 46) procedures were unsuccessful, as was a method based on the reaction of RC-12 with trans-aconitic acid, made available to us in early 1967 (personal communication, R. Strufe, Institut für Parasitologie und Veterinarmedizin, Farbenfabriken Bayer AG, 13 February 1967). This procedure worked well with pure solutions of RC-12 but was unreliable when applied to either spiked body fluids and tissue homogenates from normal monkeys or fluids and tissue homogenates from recipients of RC-12. If further studies on RC-12 are undertaken, especially those for tolerability and activity against the malarias in humans, they should be preceded by assessments of the pharmacokinetics of this compound. This effort could doubtless be served effectively by tools not available to us at the time of our studies, such as, for example, gas-liquid and high-pressure liquid chromatography and radioisotopic labeling of RC-12 at specific sites.

A preliminary report (40) on a fraction of the experiments encompassed by the above summary led others to investigate the antimalarial activities of RC-12. Two of these investigations were focused on the properties of this agent as exhibited in rhesus monkeys challenged or infected with the *B* strain of *P. cynomolgi*. One of these studies (45), concerned with the prophylactic and radical curative activities of RC-12, showed that administration of this agent in a dose of 25.0 mg/kg once or twice weekly provided complete protection against sporozoite challenge, while delivery of five, six, or seven consecutive daily doses of 25.0 mg/kg after dosage with quinine or chloroquine cured at least 11, probably 14, of 15 established infections. From these results, the authors concluded that RC-12 had greater potential as a prophylactic agent and less as a radical curative agent than was indicated in our preliminary report. This conclusion has to be accepted with reservation because of differences in design and execution of the respective experiments. In their prophylactic study, RC-12 was administered not on the day of challenge or on days 1 to 6 after challenge (40, and as reported here), but once or twice weekly for 3 weeks before

inoculation and 3 to 16 weeks thereafter. In their radical curative study, RC-12 was administered not at the same time as or before chloroquine (40, and as reported here), but subsequent to dosage with quinine or chloroquine, with no specification of the time that separated treatment courses.

The second of the studies on *P. cynomolgi* (22) was directed toward determining whether delivery of RC-12 directly to the mosquito (*Anopheles maculatus*) or indirectly via feeding on a treated monkey would affect the sporogonic cycle. The results showed that no aspect of sporogony was affected when mosquitoes were fed on sucrose solutions containing 0.1, 0.5, or 1.0% RC-12 immediately after engorgement of these insects with blood of a monkey with gametocytemia and for 12 to 16 days thereafter. In sharp contrast, oocyst formation was either completely blocked or blocked at an early stage of development when mosquitoes were fed on monkeys with substantial gametocytemias 4 to 96 hours after administration of RC-12 in a single dose of 25.0 mg/kg. The latter demonstration not only broadened the dimensions of the antimalarial properties of RC-12, but suggested that at least one of these properties could be attributed to a metabolite formed by the monkey and that this metabolite persisted in effective concentrations for 4 days after dosage. The latter suggestion, together with the persistence of protection against challenge with sporozoites for at least 6 days after the end of a 9-day dosage course (Table 4), highlights the need for systematic study of the physiological disposition of RC-12.

The preliminary report referred to previously also led to three investigations of the capacity of RC-12 to prevent malarial infections in mice. In one study (27), a dose of 400 mg/kg administered subcutaneously in an oleaginous vehicle provided protection at an unspecified level against infections with sporozoites of the 17X strain of *Plasmodium berghei yoelii*. In a second study (9) with sporozoites of the same strain, a dose of 200 mg/kg administerd intraperitoneally in saline provided no protection. In the third study (24), a dose of 300 mg/kg administered subcutaneously in saline provided questionable protection (recorded as ±) against infections with *Plasmodium yoelii nigeriensis*. The most that can be concluded from these evaluations is that RC-12 has limited, if any, activity against the early preerythrocytic stages of these murine plasmodia. Based on previous experience (31, 32), the difference between the performances of RC-12 against these plasmodia and *P. cynomolgi* was not unexpected.

It is clear from the observations summarized in this report, as well as those of others referred to above, that the spectrum of activity of RC-12 against *P. cynomolgi* infections in rhesus monkeys is very similar to that of primaquine (34), which since 1954 has been used widely and successfully for radical cure of infections with *P. vivax* (1) and less widely and successfully for prevention of infections with this plasmodium and *P. falciparum* (13, 25, 44). Both compounds are active against the early developmental tissue forms of this simian parasite, thereby according protection against infections resulting from inoculation with sporozoites. Measured by protection provided by either one or two doses administered on various days of the incubation period, both are more active against sporozoites and their immediate progeny than against the older developmental (preerythrocytic) forms. Both RC-12 and primaquine are active against the secondary or persisting tissue forms, thereby effecting cure of established sporozoite-induced infections. Both have limited activity against the blood stages of *P. cynomolgi*; thus, attainment of radical cure requires concomitant administration of a blood schizonticide, such as chloroquine. Both inhibit sporogony (22, 23).

Measured by size of dose required for the same level of accomplishment, RC-12 is much less active than primaquine. In a 9-day prophylactic regimen, the daily doses of RC-12 and primaquine required to protect 50% of the inoculees (PD_{50}) and the 95% confidence limits of these PD_{50}s (17) were 5.6 (4.5 to 7.0) and 0.5 (0.38 to 0.63) mg/kg, respectively. In a 14-day curative regimen, the daily dose of RC-12 required to eradicate 50% of established infections (CD_{50}) and the 95% confidence limits of this CD_{50} (17) was 6.6 (4.85 to 8.9) mg/kg. The corresponding CD_{50} and confidence limits for primaquine in a 7-day regimen were 0.38 (0.33 to 0.43) mg/kg. Thus RC-12 is but 1/10th as active as primaquine in preventing infections and no more than 1/17th as active as the latter compound in effecting radical cure.

There are both quantitative and qualitative differences between the toxicities of RC-12 and primaquine for the rhesus monkey. With respect to size of lethal dose, RC-12 is approximately 1/10th as toxic as primaquine. The daily doses of these agents that proved lethal to 50% of recipients (LD_{50}) and the 95% confidence limits of these doses (17) were 110 (93 to 130) mg/kg for a 15- to 17-day RC-12 regimen and 10 (7.6 to 12.5) mg/kg for a 7-day primaquine regimen. At the lethal dose level, RC-12 evoked central nervous system stimulatory and depressing reactions and moderate grade hepatotoxicity, with no evidence of hyperbilirubinemia. Sublethal doses of RC-12 evoked no outward manifestations of toxicity and, at the discrete level, only low-grade, readily reversible hepatotoxicity. Primaquine evoked a more complex group of reactions (2, 34), most of which were evident in recipients of one-eighth of the lethal dose, but became more intense as the dose was increased to the lethal level. These reactions included abdominal cramping, malaise, methemoglobinemia, anemia, reticulocytopenia, leukopenia with granulocytopenia, general suppression of bone marrow activity, and progressive manifestations of hepatotoxicity, including bilirubinemia, with widespread central lobular necrosis of the liver as a terminal event.

Relating the PD_{50}s and CD_{50}s of RC-12 and primaquine to the LD_{50}s as LD_{50}/PD_{50} and LD_{50}/CD_{50} produces a form of therapeutic index which suggests that there is little to choose between the potentials of these agents for prophylaxis or radical cure. The LD_{50}/PD_{50} indexes were approximately 20 for both agents. The LD_{50}/CD_{50} indexes were ca. 17 for RC-12 and 26 for primaquine.

The above assessment implies that RC-12 might come close to being a match for primaquine but could not be considered superior to this 8-aminoquinoline. This notwithstanding, the investigators at the Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, whose early interests in RC-12 had led to major studies on the properties of RC-12 NDS, were still interested in evaluating this pyrocatechol derivative for tolerability and antimalarial activities in human volunteers. There were at least three reasons for their continuing interest. First, RC-12 was the only non-8-aminoquinoline evaluated for activity against *P. cynomolgi* infections in rhesus monkeys over a period of 20 years that had the capacity to prevent infections and effect radical cure (40). Second, RC-12 evoked a narrower spectrum of toxic reactions than did primaquine, and these reactions to RC-12 were characterized by steeper dose-response curves. Third, since RC-12 appeared to be free of hematotoxicity, it seemed likely that its application, unlike that of primaquine, would present no special hazard to recipients with glucose-6-phosphate dehydrogenase defi-

ciency. Accordingly, plans were made in late 1967 for evaluating the toxicity and prophylactic and radical curative activities of RC-12 in prisoner volunteers at the U.S. Penitentiary, Atlanta, Ga., and submitted to the National Institutes of Health review group that was responsible for approval of trials of new agents in humans. The proposed study was disapproved, reportedly because primaquine met identifiable needs for prophylaxis and radical cure of infections with *P. vivax* so effectively that commitment of human volunteers to evaluations of an agent comparable to this 8-aminoquinoline with respect to scope of activity and efficacy could not be justified.

Some 6 years later, concerns with the performance of primaquine in Vietnam (32) led those responsible for the U.S. Army Malaria Research Program to sponsor limited appraisals in human volunteers of the tolerability of RC-12 and its capacities to protect against infections with sporozoites of the Chesson strain of *P. vivax* and cure established infections with this strain (6). The largest dose of RC-12 administered, 10.0 mg/kg daily for 7 days, comparable to that which regularly protected against and cured infections with *P. cynomolgi*, was fully tolerated. Application of this dose in a conventional prophylactic regimen neither prevented infections nor delayed their onset in three of three volunteers, nor did it cure established infections in two of two volunteers when administered concomitantly with chloroquine.

To some, the failure of RC-12 to prevent or cure infections with *P. vivax* when administered in a dose very close to that which regularly prevents and cures infections with *P. cynomolgi* sufficed to end interest in this agent and the chemical family to which it belongs (5, 24). Our reaction is somewhat different. RC-12 is unique among non-8-aminoquinolines with respect to providing complete protection against and effecting cure of infections with *P. cynomolgi* at well-tolerated doses and interrupting the sporogonic cycle. To this point, the performances of test compounds against infections with this simian plasmodium and those against infections with the Chesson strain of *P. vivax* have been essentially identical. In view of this, it would seem premature to place RC-12 in the category of inactive antimalarial agents without first determining whether the dichotomy between the activities exhibited against infections with *P. vivax* and *P. cynomolgi* rests on differences in physiological disposition in humans and rhesus monkeys. The inactivity of RC-12 against infections with *P. vivax* might be due to poor absorption of the compound when administered as the naphthalenedisulfonate, uncommonly rapid renal clearance, speedy conversion of absorbed compound to an inactive metabolite, failure to convert the parent agent to an active metabolite, or poor localization of parent compound or active metabolite in the liver. Attention to such parameters might point the way to effective application of RC-12 per se or its metabolites, or even an appropriately designed congener, against infections with *P. vivax*. At the least it should insure against premature shelving of interest in the pyrocatechols comparable to that which occurred in 1929 after inappropriate evaluation of the activity of Dimeplasmin, a close structural relative of RC-12 (40).

Finally, identification of the factor(s) responsible for the negative performance of RC-12 against infections with *P. vivax* is of special importance to the continuing use of infections with *P. cynomolgi* in the search for more effective and generally useful tissue schizonticides. This use dates from 1948, when it was shown that infections with sporozoites of this simian plasmodium in rhesus monkeys and those of the Chesson strain of *P. vivax* in human volunteers responded in an essentially identical manner to such drugs as quinine, chloroquine and other 4-aminoquinolines, chlorguanide, pamaquine, pentaquine, isopentaquine, and primaquine (36). Since that time, 201 8-aminoquinolines and 187 non-8-aminoquinolines have been evaluated for activity against infections with *P. cynomolgi* (31, 32), with the assumption that the results of these assessments carried over to infections with *P. vivax*. Unless the dichotomy between the activities of RC-12 can be explained, confidence in the predictive value of results acquired in the simian infection must be qualified. Prompt resolution of this dichotomy is a matter of considerable urgency for, because of current obstacles to pursuing studies in human volunteers, even greater reliance must be placed on results obtained in lower animal test systems if the search for better tissue schizonticidal drugs is to be pursued productively.

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