SPOROZOITE-INDUCED *PLASMODIUM CYNOMOLGI* INFECTIONS IN CAPTIVE BORN *MACACA FASCICULARIS*

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INTRODUCTION

The simian malaria parasite, *Plasmodium cynomolgi* has been extensively used in malaria research for testing of antimalarial drugs (Kinnamon *et al.*, 1975; Schmidt *et al.*, 1977b; Davidson *et al.*, 1981) and for studies of persistent exoerythrocytic stages of the malaria life cycle (Krotoski *et al.*, 1981). It has been useful, since the life cycle is similar in many respects to that of human *P. vivax*. Although a variety of nonhuman primates may be infected with *P. cynomolgi* by trophozoite or sporozoite inoculation (Schmidt *et al.*, 1977a) only rhesus monkeys (*Macaca mulatta*), which develop parasitemias of sufficient magnitude and uniformity to be a useful model for drug testing, have been routinely used. Recent scarcity and the expense of young rhesus has impeded malaria research and drug testing.

A breeding colony of rhesus and cynomolgus monkeys (*Macaca fascicularis*) has been established in Bangkok at Armed Forces Research Institute of Medical Sciences for research purposes. Cynomolgus production has been quite successful and captive born monkeys have become available for research in substantial numbers. Infection of wild caught cynomolgus monkeys with *P. cynomolgi bastianellii* was studied (Schmidt *et al.*, 1977a) but results were not encouraging. Because the cynomolgus monkey is a natural host for *P. cynomolgi* as well as other simian malarias (Coatney *et al.*, 1971), previous natural exposure could have modified the infection response in wild caught monkeys. The possibility of substituting captive born, malaria-naive cynomolgus for rhesus in the radical curative antimalarial drug testing program prompted the present study. In addition, effects of larger sporozoite inocula and splenectomy were investigated in this animal model.

The opinions or assertions contained herein are the private views of the authors and are not to be construed as opinions of the Department of Defense or the Department of the Army.

MATERIALS AND METHODS

Eleven male and 7 female captive born cynomolgus monkeys whose weights ranged from 1.9 to 2.9 kg were used. Their parents' origin was Peninsular Malaysia. The primate colony was located in Bangkok, Thailand, where malaria vector mosquitoes are absent. A commercial diet (Wayne Laboratory Animal Diets, Chicago, I11), fresh fruit 3 times weekly, and water *ad libitum* were provided. Housing was in individual cages measuring 70 × 62 × 70 cm. Ketamine (Parke-Davis, Morris Plains, NJ) and acepromazine (Ayerst Laboratories Inc. New York, NY) were used for anesthesia for surgery or mosquito feeding. Splenectomy was performed 14 to 87 days prior to sporozoite inoculation.

The monkey-mosquito-monkey cycle was maintained by feeding *Anopheles dirus* mosquitoes on sporozoite infected rhesus monkeys. Mosquitoes were fed on the second peak of the primary attack when both male

and female gametocytes were present. The malaria strain used was *P. cynomolgi bastianellii*; the complete methodology for the model has been outlined (Schmidt *et al.*, 1963; Davidson *et al.*, 1976). Estimates of oocyst and sporozoite production in mosquitoes were made on post-feeding days 5 and 13, respectively. For sporozoite harvest and inocula preparation, mosquito thoraces were isolated by dissection and ground in saline. In the standard rhesus model for radical curative testing against persistant-tissue stages of *P. cynomolgi* an individual inocula of $0.5 - 2.0 \times 10^6$ sporozoites has been used. In this study, the standard dose or a higher ($0.3 - 1.0 \times 10^7$) dose of sporozoites was given. Rhesus monkeys on a concurrent drug testing project served as sporozoite infectivity controls.

Eighteen cynomolgus were divided into three groups and inoculated with sporozoites as follows : 4 were given a standard (low) dose; 8 were given a higher (high) dose; and 6 were splenectomized and given the higher dose. Following intravenous infection with sporozoites, thick and thin blood films, obtained by puncturing the marginal ear vein with a lancet, were made daily until the primary attack phase ebbed and then twice weekly for at least 120 days. Values were expressed as number of parasites per cubic millimeter of blood (p/c.mm). Preinfection and weekly blood counts were continued until the end of the primary attack. Parasite relapse due to tissue stages, following clearance of all blood forms, was tested in two monkeys by administration of the blood schizonticide, chloroquine phosphate, at 10 mg/kg (salt) with or without a subcurative dose of the tissue schizonticide, primaquine phosphate. Drug administration was via nasogastric tube for a period of seven consecutive days.

RESULTS

Parasitemias developed at an average of 7.9 days. Mean parasitemic data for the low dose, high dose and high dose-splenectomized groups are shown in Table 1. Data from six rhesus mosquito feeders infected concurrently in an ongoing drug screening program has been included for comparison. Although low and high dose infection of intact cynomolgus resulted in a persistant parasitemia, the maximum parasitemia was markedly less than in

Table 1

Effect of sporozoite dose and splenectomy on parasitemia persistance and level.

Group (No.)	Sporozoite dose	Postinfection day of patency	Mean maximum parasitemia (Parasites/ c.mm)	Mean # positive blood films/total examined (%)	Mean parasitemia from day 42 to 120 (Parasites/ c.mm)
M fas (4)	$0.8\text{-}1.7 \times 10^6$	8.3	13,257	41/69 (59)	16
M fas (8)	$3\text{-}10 \times 10^6$	8.3	32,026	47/74 (63)	44
M fas-S* (6)	$3\text{-}4 \times 10^6$	7.7	102,159	82/90 (91)	571
M mul (6)	$0.8\text{-}2.0 \times 10^6$	7.4	543,417	17/17 (100)	ND

M fas = *Macaca fascicularis;* M mul = *Macaca mulatta*
*-S = Splenectomized prior to infection
ND = Not done

splenectomized cynomolgus or intact rhesus and the percent of positive blood films was lower. Mean parasitemia in the splenectomized group was much higher 41-120 days after infection than in the 2 intact groups of cynomolgus. Fig. 1 shows the mean logarithmic parasitemia curve for each group through post infection day 40. The low and high sporozoite dosed intact cynomolgus groups had similar mean parasitemias over this 40 day observation. The mean parasitemia for the splenectomized group was consistently higher and these higher levels persisted longer. Mean preinfection red blood cell count for all groups was 7.3×10^6. The mean red blood cell counts were 6.2 and 6.3×10^6, respectively, for the low and high groups of intact cynomolgus but were 4.9 and 4.6×10^6, respectively, for rhesus and the splenectomized cynomolgus. Leukocytes (WBC) and the number of lymphocytes at preinfection, 2 weeks, and 3 weeks following sporozoite inoculation are shown in Table 2. There was a 248% increase in lymphocytes from preinfection to 2 weeks post infection in the splenectomized group. In this group, mean WBC count from post-inoculation day 12 through 22 was 30,464. Over a 6 week post-splenectomy period in 3 cynomolgus from a different study that were not given sporozoites there was a maximum lymphocyte increase of 5%.

Sporozoite production from mosquitoes fed on the 3 cynomolgus groups varied with the surgical status of the monkey. Gametocyte production was poor in the intact monkeys, with only 5 of 12 intact monkeys producing gametocytes. Male gametocytes were

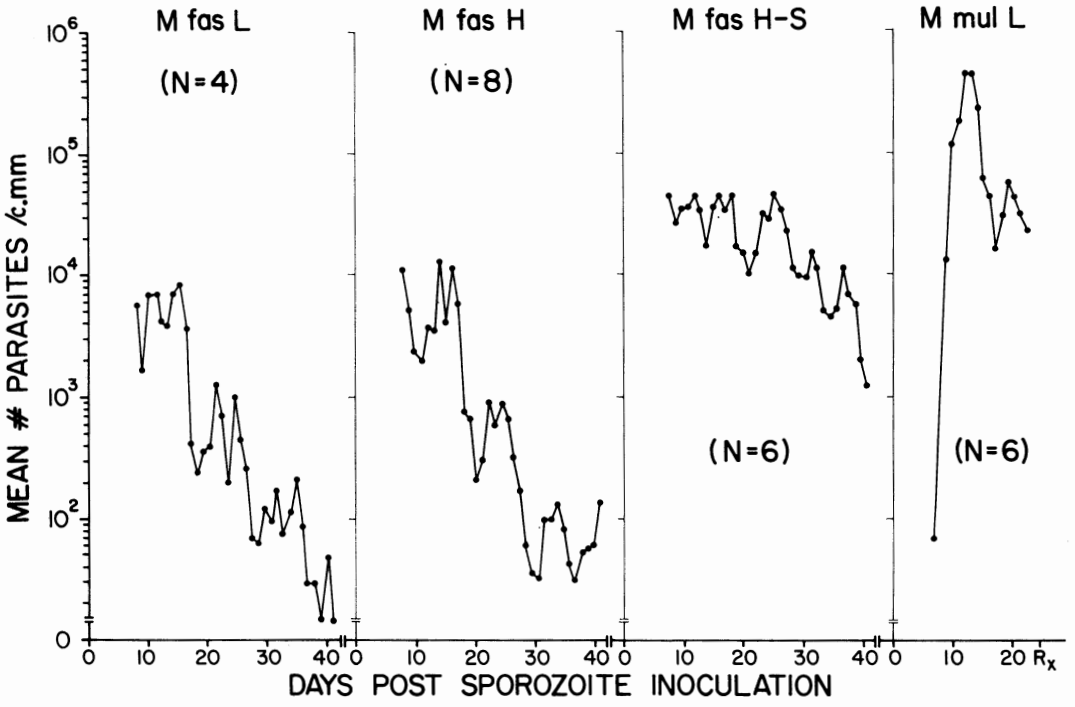

Fig. 1—Mean parasitemia of *Macaca fascicularis* (M fas) or *Macaca mulatta* (M mul) during first 40 days following low (L) 0.8 - 2.0 $\times 10^6$ or high(H) 0.3 - 1.0 $\times 10^7$ sporozoites intravenously. Group M fas H-S was splenectomized prior to infection.

Table 2

Effect of splenectomy on leukocytes and lymphocytes at preinfection, 14 and 21 days postinfection.

Group (Number)	Preinfection		14 Days Postinfection		21 days Postinfection	
	Total leukocytes	Lymphocytes	Total leukocytes	Lymphocytes	Total leukocytes	Lymphocytes
M fas (4) L*	11,550	6,953	12,150	9,455	9,800	5,680
M fas (8) H**	13,963	6,414	14,538	9,021	14,175	10,159
M fas-S*** (6) H	11,850	7,607	32,250	26,483	28,500	22,412
M mul (6) L	ND	ND	11,383	ND	12,217	ND

M fas = *Macaca fasicularis;* M mul *Macaca mulatta*
*L = 0.8-2 × 10^6 sporozoite Inoculum
**H = 0.3-1.0 × 10^7 sporozoite Inoculum
***-S = Splenectomized prior to infection

Table 3

Oocyst and sporozoite production from mosquitoes fed on splenectomized *Macaca fascicularis.*

Monkey #	Post patent days when mosquitoes were fed	Oocyst positive mosquitoes/ total	Mean oocyst No.	Mean oocyst diameter (μ)	Sporozoite positive mosquitoes/ total
AF-18	11, 12, 13, 15, 22	22/25	59	24.6	25/25
AF-9	14, 15	5/8	1.7 ACF	39.4 ACF	ACF
AF-16	17, 18	10/10	91	53.3	10/10
AF-13	16,17	10/10	91	43.6	8/8
AF-11	17,18	7/10	95	46.1	9/10
AF-14	10	4/5	100	NM	5/5

ACF = Air conditioning failure
NM = Not measured

seen in only two of these five. Sporozoite production resulted in only one out of six mosquito feedings on intact monkeys. All splenectomized monkeys produced abundant male and female gametocytes. On the days of mosquito feeding, gametocytemias ranged from 500 to 23,500/c.mm with a male to female ratio ranging from 1 : 3 to 1 : 13. Mosquitoes were fed from post patent day 11 to 20. All lots of mosquitoes fed on the splenectomized cynomolgus developed sporozoites or oocysts (Table 3). Feeding mosquitoes on post patent day 14 or later was more productive. The mean oocyst number and diameter for post patent feeding days 14-22 were 93 and 42 microns(μ) as compared to 38 and 24 microns(μ) for post patent days 11-13. An insectory air conditioning failure resulted in poor oocyst production and failure of sporozoite production from mosquito lots

which had fed on rhesus as well as on one splenectomized cynomolgus. Sporozoite densities in salivary glands of mosquitoes fed on splenectomized cynomolgus was similar to those observed in rhesus. A harvest of sporozoites from mosquitoes which fed on one splenectomized monkey (#AF-18) was used as inoculum for inducing infection in another (#AF-9). The resulting infection was indistinguishable from those induced with sporozoites harvested from mosquitoes which fed on rhesus.

Relapse potential was tested in 2 splenectomized cynomolgus to evaluate their possible response in a drug screening programme. Chloroquine was given to AF-9 following 113 days of continuous parasitemia. Following clearance of circulating forms, a relapse occurred 27 days after the last treatment. Circulating forms in AF-13 were cleared with chloroquine after only 35 days of patency. Following 11 negative days a relapse was observed in AF-13.

Resistance of tissue stages were next evaluated by giving subcurative doses (0.3 & 0.1 mg/kg) of the tissue schizonticide, primaquine, along with 10 mg/kg chloroquine to AF-13 & AF-9 respectively. Following clearance of the parasitemia these monkeys relapsed 28 and 26 days post-treatment. To terminate the infection in all monkeys chloroquine at 10.0 mg/kg and primaquine at 1.3 mg/kg were administered. All cynomolgus responded to these doses without subsequent relapse.

DISCUSSION

The intact captive born cynomolgus does not appear to be capable of substituting for rhesus in the simian radical curative antimalarial model, even when given a high inoculum of sporozoites. Although a persistent infection is established, the parasitemia level is quite low and of doubtful value in evaluating antimalarial drug activity. The possibility of maintaining a monkey-mosquito-monkey cycle in intact cynomolgus is remote becuase of inadequate gametocyte production. Probability of sporozoite production is low and occurred in mosquitoes fed on only one of 6 monkeys.

The splenectomized captive born cynomolgus was capable of sustaining a monkey-mosquito-monkey cycle. Mosquito feedings from all six monkeys produced oocysts, and an inoculum of sporozoites harvested from mosquitoes which fed on one was infective for another. Oocyst and sporozoite production from feedings on splenectomized cynomolgus was comparable to that of rhesus. It was found that feeding mosquitoes later in the post patent period, presumably after the initial immune response had subsided, was more productive as has been noted in rhesus. Splenectomy increased the severity of *P. cynomolgi* parasitemia and allowed near maximum parasite numbers on the first day of patency. There was a 37% decrease in red cell count as compared to an average decrease of 14% in intact cynomolgus. The splenectomized mean parasitemia values from day 41 to 120 were 13 times higher than the intact values and were comparable to untreated infections in rhesus (Schmidt *et al.*, 1982). During the 1st 40 days splenectomized cynomolgus parasitemia values were similar to those obtained in rhesus except for the first peak in the primary attack. Relapse potential was observed in the two tested. Considering these factors by themselves, the captive born splenectomized cynomolgus given a high sporozoite inoculum seems promising as a potential substitutere for rhesus in *P. cynomolgi* antimalarial drug studies. The mean parasitemic data, however, is misleading since two of the six splenectomized monkeys had low curves after the first three weeks of patency, which could diminish their usefulness. In addition, the disadvantages of

splenectomy must be considered before recommending this model as a supplement or substitution for rhesus. There is a general reluctance of investigators to re-use a splenectomized monkey for most kinds of studies. Specifically, these animals would not be useful for further infectious disease research. This would be a disadvantage from the standpoint of maximum utilization of primates for conservation purposes. Our laboratory will pursue the use splenectomized cynomolgus only as a supplement, to screen potentially toxic compounds before their definitive testing in rhesus.

An unexpected observation was the marked leukocytosis following patency in the splenectomized group. This consisted of an absolute lymphocytosis which average 26, 483 at 14 days post inoculation. This was not an isolated occurrence, as the mean WBC count from post-patent day 5 to 15 for all splenectomized monkeys was 30,464. Both high dose sporozoite inocula groups were within normal WBC range prior to infection, but following infection, the splenectomized group had an exaggerated response while the intact group remained normal. Since 3 other splenectomized cynomolgus which did not receive a large sporozoite inocula failed to produce a similar leukocytosis, the antigenic load of sporozoites may have been the inciting factor.

SUMMARY

Capability of captive born cynomolgus monkeys to substitute for rhesus in the *Plasmodium cynomolgi* radical curative antimalarial drug development model was examined. Eighteen monkeys divided into 3 groups were given standard or high doses of sporozoites intravenously. One group of 4 received $0.8 - 1.6 \times 10^6$ and a second group of 8 received $0.3 - 1.0 \times 10^7$ sporozoites. The third group of 6 was splenectomized and then received $3.0 - 4.0 \times 10^6$. The 2 groups of intact monkeys developed a persistant low level parasitemia; however, gametocyte production was poor. The splenectomized group developed a persistant parasitemia with a higher mean, which more closely resembled rhesus parasitemias. A high, post-patent leukocytosis consisting primarily of lymphocytes was observed in this group. Good gametocyte production resulted in the splenectomized group and oocysts were produced from all lots of *Anopheles dirus* which fed on them. Following clearance of blood forms, relapse potential was demonstrated in the 2 splenectomized monkeys tested. In this study the splenectomized captive born cynomolgus appeared to be capable of supplementing rhesus as an antimalarial drug testing model.

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