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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) Acute clinical malaria caused by <u>Plasmodium inui</u> was diagnosed in an adult fe- male cynomolgus monkey (<u>Macaca fascicularis</u>) 4 years after importation into the United States. Severe clinical disease was attributed to activation of a chronic infection by the stress associated with experimental procedures completed 2 weeks earlier. Clinical findings included severe regenerative anemia, hepatospleno- megaly, weakness, lethargy, weight loss, and anorexia. The infection was treated and successfully eliminated with chloroquine hydrochloride, administered (CONTINUED ON REVERSE)		

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20. Abstract (CONT)

intramuscularly at a dose of 5 mg/kg base given at 0, 6, 24, 48 and 72 hours. Treatment also included a blood transfusion and intensive supportive care. Naturally-occurring malarial infections in nonhuman primates are usually asymptomatic; however, this case was accompanied by severe clinical signs. Laboratory investigators using nonhuman primates should be aware of the potential for activation of latent malarial infections that may result in clinical disease. Susceptible nonhuman primates to be used in biomedical research should be screened for malaria and appropriately treated to prevent potential complications that may result from this disease.

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Acute Clinical Malaria (Plasmodium inui) in a Cynomolgus
Monkey (Macaca fascicularis)^{1,2,3,4}

Short Title: Malaria in a Cynomolgus Monkey

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²In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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Summary | Acute clinical malaria caused by Plasmodium inui was diagnosed in an adult female cynomolgus monkey (Macaca fascicularis) 4 years after importation into the United States. Severe clinical disease was attributed to activation of a chronic infection by the stress associated with experimental procedures completed 2 weeks earlier. Clinical findings included severe regenerative anemia, hepatosplenomegaly, weakness, lethargy, weight loss, and anorexia. The infection was treated and successfully eliminated with chloroquine hydrochloride, administered intramuscularly at a dose of 5 mg/kg base given at 0, 6, 24, 48 and 72 hours. Treatment also included a blood transfusion and intensive supportive care.

Key Words | Malaria, Macaca fascicularis, Plasmodium inui

Malaria in man and nonhuman primates is caused by protozoa of the genus Plasmodium. Infections result from the introduction of sporozoites into the primate host from the bite of infected mosquitoes. Nonhuman primates imported from endemic malarial regions are frequently found to be infected (1,2). These malarial infections are usually chronic and subclinical in nonhuman primate hosts, and rarely result in clinical disease (1,2) unless the animal is splenectomized, immunosuppressed, or otherwise stressed (3).

Wild-caught cynomolgus monkeys (Macaca fascicularis) are commonly infected with malaria, caused by Plasmodium coatneyi, P. cynomolgi, P. fieldi, P. inui, and/or P. knowlesi (4). The incidence of these natural infections has ranged between 2 and 43% (5-7) in imported cynomolgus monkeys. Natural and experimental malarial infections in cynomolgus monkeys are generally subclinical and of mild pathogenicity (1,8). Severe malaria resulting from natural infection has been reported only once and was attributed to P. inui (9). However, severe malaria in cynomolgus monkeys has infrequently resulted from experimentally induced infections with P. cynomolgi (10), P. inui (9), and P. knowlesi (11,12). One experimental case occurred in a splenectomized monkey (12), but the remainder involved cynomolgus monkeys with intact spleens and uncompromised immune defense mechanisms.

The purpose of this report is to describe a case of acute clinical malaria in a cynomolgus monkey. This is the first report of clinical malaria in a natural nonhuman primate host where activation of the infection was associated with the stress of laboratory procedures.

Case Report

Clinical History: A wild-caught, 2.7-kg adult female cynomolgus monkey trapped near Kuala Lumpur, Malaysia, was obtained from a commercial source⁷ in December 1976. After importation, the monkey was housed indoors in an individual cage for 4 years with no research utilization or observation of any clinical disease.

In December 1980, the monkey was placed on a 14-day experimental protocol involving surgical implantation of catheters, experimental Streptococcus pneumoniae infection and hyperalimentation treatment. A complete blood cell count (CBC) performed prior to the experiment was within normal limits (Table 1). On day zero of the protocol, intravenous catheters were surgically implanted and a specially designed leather jacket fitted to the monkey to protect the catheters (13). On day 8, 5×10^8 pneumococcal organisms were administered intravenously and the infection was untreated for 48 hours. On day 10, 40 ml of blood were drawn for lymphocyte transformation assays and other analytical tests; the volume was replaced with 40 ml of whole blood collected from a clinically normal cynomolgus monkey blood-donor. Also starting on day 10, 150,000 units each of penicillin G procaine and penicillin G benzathine⁸ were administered intramuscularly and this treatment was given daily for the next 6 days. On day 14, the monkey was removed from the tethered jacket and the catheters surgically removed. The monkey appeared clinically normal at this time and a CBC from the previous day reflected only a slightly depressed hematocrit (Table 1). On day 22, mild anorexia was noted, which gradually progressed in severity during the next 7 days. On day 29 the monkey was lethargic and completely anorectic.

⁷Charles River Primate Research Corporation, Port Washington, NY.

⁸Wyeth Bicillin Fortified^R, Wyeth Laboratories, Inc., Philadelphia, PA.

Physical Findings: Examination on day 29 revealed extremely pale mucous membranes, emaciated body condition, palpable hepatosplenomegaly, weakness, slight pyrexia of 39.2°C, and increased respiratory and heart rates. A grade III/VI holosystolic ejection murmur near the base of the right heart was also detected. Blood samples were drawn for a CBC and selected serum chemistries.

Laboratory Findings: Hematologic results on day 29 revealed severe regenerative anemia with depressed hematocrit, hemoglobin, and red blood cell count⁹ (Table 1). Severe anisocytosis, marked polychromasia, nucleated red blood cells, and neutrophilic leukocytosis were observed. Eight percent of the red blood cells were infected with malarial parasites at a concentration of 1.8×10^5 parasites/dl blood. All stages of developing trophozoites and schizonts were present in the erythrocytes (Figure 1). Doubly and triply infected cells were observed, as well as monocytes containing malarial pigment. The malaria was identified as P. inui based upon morphologic characteristics.⁴ Blood urea nitrogen, serum creatinine, and serum electrolytes were within normal limits. Serum total protein was low normal at 6.8 g/dl.

Treatment: Antimalarial therapy was begun immediately using chloroquine hydrochloride¹⁰ at a dose of 5 mg/kg chloroquine base administered intramuscularly followed by the same dose repeated at 6, 24, 48 and 72 hours after the initial treatment for a total dose of 25 mg/kg. Antibiotic therapy to prevent secondary infections was instituted using 150,000 units each of penicillin G procaine and penicillin G benzathine intramuscularly and repeated daily for the next 10 days. Supportive therapy included the administration of parenteral B-vitamin complex,¹¹ ad libitum oral glucose-electrolyte solution¹² and subcutaneous electrolyte fluids.¹³

⁹Coulter Counter^R Model ZBI, Coulter Electronics, Inc., Hialeah, FL.

¹⁰Aralen^R Hydrochloride, Winthrop Laboratories, New York, NY.

¹¹Solu-B-Forte^R (S-B-F^R), The Upjohn Company, Kalamazoo, MI.

¹²Life-Guard^R, Norden Laboratories, Lincoln, NE.

¹³Lactated Ringers Injection, USP, Cutter Laboratories, Berkeley, CA.

Clinical Course: Twenty-four hours after treatment was started, the anemia worsened (Table 1). The animal was noticeably weaker and body temperature had decreased to 37.2°C. Antimalarial and supportive therapy was continued with the addition of supplemental oral feedings of glucose/electrolyte solution by nasogastric tube.

Forty-eight hours after treatment was begun the monkey was extremely weak, body temperature had decreased to 36.3°C, and the anemia had worsened further (Table 1). Serum chemistry values showed hypoalbuminemia (2.2 g/dl), hypoproteinemia (5.0 g/dl), and increased total bilirubin (0.6 mg/dl), serum glutamic oxaloacetic transaminase (55 U/liter), serum glutamic-pyruvic transaminase (84 U/liter) and serum alkaline phosphatase (573 U/liter). A blood transfusion was administered using 75 ml of cynomolgus monkey whole blood collected in anticoagulant solution¹⁴. Improvement was evident the next day; by day 35 the monkey appeared clinically normal. The heart murmur decreased in intensity following the blood transfusion and was absent one week after originally detected. No malarial parasites were observed on thin blood smears after completion of the 3-day chloroquine therapy and hematologic parameters gradually returned to normal over the next 6 weeks (Table 1).

Follow-up: Thick and thin blood smears were examined monthly over the next 5 months and were all found to be negative for malarial parasites. Six months after the clinical disease, the monkey was immunosuppressed to determine if a chronic latent malarial infection might still persist. The immunosuppressive regimen consisted of 4.4 mg/kg of prednisolone acetate¹⁵ given daily for 3 weeks by intramuscular injection. No parasites were observed in thin or thick blood smears examined during this regimen or during a 6 month follow-up period.

¹⁴ACD, A. J. Buck and Sons, Inc., Cockeysville, MD.

¹⁵Sterile Prednisolone Acetate USP, D-M Pharmaceuticals, Rockville, MD.

A review was made of the lymphocyte transformation assay data performed on day 10 of the pneumococcal infection experiment. The responses of peripheral blood lymphocytes in whole blood cultures to mitogen stimulation with concanavalin A, phytohemagglutinin, and pokeweed mitogens were measured by standard micro-culture technique (15). Stimulation indexes for each of these mitogens were used to assess cell-mediated immune functions (15). Values for the monkey described in this case report as well as five other similarly treated monkeys were compared to those for six noninfected control monkeys (Table 2). The six monkeys with the acute pneumococcal infection were shown to have much lower lymphocyte stimulation indexes than the six noninfected control monkeys (Table 2).

The two cynomolgus monkeys used as blood donors, one during the pneumococcal experiment and a second during the acute malaria, were rescreened for malaria 3 months later. No malarial parasites had been detected on several CBC examinations on either monkey during the previous 2 years. The first donor, a wild-caught male imported⁶ from Malaysia, was found on thick blood smears to have low-level parasitemia of 600 malarial parasites/dl. The monkey used as the blood donor for the second transfusion was negative for hemoparasites on both thin and thick smear examination.

It was later found that a matched control monkey from the pneumococcal experiment had also received a 40 ml blood transfusion from the first donor monkey later found to have a chronic malaria infection. This control monkey was subjected on the same dates to the same experimental protocol as the case described here, except that it was not infected with S. pneumoniae. This control monkey did not develop any clinical signs or abnormal CBC parameters during or after

the experiment and no malarial parasites were observed on several CBC examinations during the 6 months following the experiment.

Discussion

Most Plasmodium spp. infections in cynomolgus monkeys have been described as chronic and asymptomatic (1,2), with acute clinical malaria only rarely reported. The reasons for development of acute clinical malaria in the few cases previously reported have not been clearly explained. Two reports suggest that some degree of stress or other immunosuppression contributed to the development of clinical disease (9,12). One description (9) of fatal disease attributed to a naturally acquired infection of P. inui was observed shortly after shipment; and the stress associated with shipment may have been a contributing factor to development of symptomatic infection. The second report (12) described a case of acute fatal malaria that resulted from an induced infection following splenectomy. The lack of a spleen and its associated protective functions (14) undoubtedly contributed to the severe disease that developed.

Experimental stress appears to have contributed to the clinical malaria described in this case report. This stress resulted from experimental manipulation that involved two surgical procedures, 2 weeks in a tethered jacketing system, and an experimentally induced acute pneumococcal infection. Immunosuppression during the acute pneumococcal infection was demonstrated for the monkey described here as well as five other similarly treated monkeys when compared to the six noninfected controls. The depressed immune functions found to occur during the acute pneumococcal infection may have allowed a rapid expansion of a low-level parasitemia, with the subsequent development of clinical

malaria. The control monkey that was treated in the same manner as this case, except for the pneumococcal infection, did not develop any clinical signs or abnormal blood parameters. Suppression of the normal immune defense mechanisms thus appears to have been a significant contributing factor in the development of clinical disease in this case.

Another report (11) of clinical malaria in cynomolgus monkeys suggested that an intraspecies variation in susceptibility to malaria exists depending upon the geographic origin of the monkeys. It was demonstrated that experimental P. knowlesi infections caused uniformly fatal disease in cynomolgus monkeys of Malayan origin, but only mild chronic infections in cynomolgus monkeys of Phillipine origin. However, this variation in intraspecies susceptibility does not account for the fact that Malayan monkeys often harbor asymptomatic P. knowlesi infections. It is not known if such an intraspecies variation in susceptibility to P. inui exists.

The source of the malaria infection causing the clinical disease in our case was probably from a chronic pre-existing infection obtained in the Malaysian jungle prior to importation 4 years previously. P. inui, the species identified in this case, is the most widely distributed and most common malaria species found in cynomolgus monkeys and has been isolated from all areas of their natural habitat (12). The chronic asymptomatic persistence of the infection for 4 years would not be considered unusual, as naturally occurring quartan malarial infections such as P. inui persist as low-level parasitemias for long periods (4). Cynomolgus monkeys housed in our colony for over 7 years have been found with chronic P. inui infections; furthermore, induced P. inui infections have been reported to persist in rhesus monkeys for up to 14 years (16). In addition to a naturally acquired infection,

there is also the possibility that the infection in this case may have originated from or been enhanced by the blood transfusion received during the experimental protocol on day 10 from the blood donor monkey later found to have a chronic malarial infection. However, the matched control monkey that also received an identical blood transfusion from this donor monkey on the same day did not develop clinical disease or abnormal blood parameters.

The clinical signs observed in this case were similar to those observed in other nonhuman primates with acute severe malaria resulting from natural or experimental Plasmodium infections (1,11,17). Most of the clinical signs observed could be attributed to the severe anemia that resulted. The transient heart murmur was attributed to the anemia as well. The hepatosplenomegaly observed is also a common feature of acute and chronic malaria.

Chloroquine hydrochloride was used as the antimalarial drug because of its wide acceptance as a standard schizonticidal drug for treatment of acute and chronic human malaria. No previous reports of treatment of acute malaria in cynomolgus monkeys exists; therefore, a dosage was extrapolated from the manufacturer's human pediatric recommendations (18). The total dose of 25 mg/kg chloroquine base divided into five doses over 3 days was successful in completely eliminating the infection, as determined by repeated negative blood smears. No adverse effects attributable to the drug were observed. The efficacy of this therapeutic regimen was further supported by negative thick blood smears following the immunosuppressive corticosteroid regimen administered after full recovery. Such immunosuppression has been shown to cause reactivation of latent malarial infections not detectable on thick blood smear examination (12).

The detection and control of malaria in laboratory nonhuman primates should include initial screening of all potentially infected animals by microscopic examination of thick blood smears. Potentially infected animals include not only wild-caught nonhuman primates imported from regions with endemic malaria, but also the domestically reared offspring of such animals that may have acquired congenital infections. In addition, laboratory-acquired infections in primates could potentially result from blood-contaminated equipment, blood transfusions, or tissue transplantations from infected individuals.

Careful identification of the parasite species involved in acute or chronic malaria is important. The differential diagnosis of blood parasites in wild-caught cynomolgus monkeys should include not only the five naturally occurring malarial species, but also Hepaticystis semnopithecii and Entopolypoides macaci. The latter two blood sporozoans are commonly found in cynomolgus monkeys from Southeast Asia; however, no clinical sequelae have been associated with these infections (3).

Treatment of infected animals should initially consist of a schizonticidal drug such as chloroquine hydrochloride to eliminate the parasitemia. In the case of two relapse malarias found in cynomolgus monkeys, P. cynomolgi and P. fieldi, this initial treatment should be followed by additional treatment with primaquine to eliminate persistent hepatic schizonts. Follow-up examinations should then be performed on all animals to ensure that treatment successfully cleared the infection.

This case of severe clinical malaria demonstrates the potential for a normally asymptomatic Plasmodium infection to cause serious clinical disease in a natural host subjected to laboratory

stress. Clinicians and investigators using potentially infected nonhuman primates must be aware of this possible development of clinical malaria. Appropriate preventive measures and treatment should be instituted to safeguard the health of susceptible laboratory animals, as well as to prevent malaria from complicating the results of biomedical research.

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Table 1

Blood values before, during and after acute clinical malaria in a cynomolgus monkey

Parameter	Values by days										
	Acute disease						Convalescence				
	-18	13	29	30	31	32	41	57	73		
Hematocrit (%)	38.4	32.0	15.1	12.0	9.7	29.4	30.4	34.2	42.1		
Hemoglobin (g/dl)	13.4	9.8	4.4	ND ^a	3.2	8.3	9.1	10.6	11.8		
RBC (no. x 10 ⁶ /mm ³)	5.69	5.04	2.2	1.8	1.52	3.86	4.41	5.29	6.65		
WBC (no. x 10 ³ /mm ³)	9.9	5.8	14.6	13.4	11.9	4.1	3.4	4.7	8.2		
Band neutrophils (%)	-	-	4	8	3	-	-	-	-		
Segmented neutrophils (%)	27	39	51	34	40	52	22	27	54		
Lymphocytes (%)	69	61	41	53	46	46	61	56	36		
Monocytes (%)	2	-	3	-	2	2	14	13	8		
Eosinophils (%)	2	-	-	-	-	-	2	4	2		
Basophils (%)	-	-	1	-	-	-	-	-	-		
Nucleated RBC/100 WBC	-	-	1	1	3	-	-	-	-		
Reticulocytes (%)	ND	ND	ND	ND	10.0	ND	5.2	2.1	ND		
Mean corpuscular volume (μ ³)	66	64	67	65	64	75	69	64	62		
Mean corpuscular hemoglobin conc'n (%)	34.9	30.6	29.1	ND	33.0	28.2	29.9	31.0	28.0		
Mean corpuscular hemoglobin (pg)	23.5	19.4	20.0	ND	21.1	21.5	20.6	20.0	17.7		
Serum total protein (mg/dl)	ND	7.6	6.8	6.4	5.0	6.9	7.4	7.8	ND		
Chloroquine treatment			+	+	+	+	+	+			

^aND = Not done

Table 2

Effect of experimental *S. pneumoniae* infection in cynomolgus monkeys during hyperalimentation studies: lymphocyte transformation mitogen stimulation indexes^{a, b} from whole blood cultures

Mitogen	Mean stimulation index	
	Controls (n = 6 monkeys)	Infected(% of controls) (n = 6 monkeys)
Pokeweed mitogen (PWM)	30.74	4.28 (13.9)
Phytohemagglutinin (PHA)	15.34	5.62 (36.5)
Concanavalin A (ConA)	5.06	1.95 (38.5)

^a Stimulation index = $\frac{\text{dpm in mitogen-stimulated lymphocytes}}{\text{dpm in nonstimulated lymphocytes}}$

^b Cultured with 4 $\mu\text{g/ml}$ PWM, 4 $\mu\text{g/ml}$ PHA, or 10 $\mu\text{g/ml}$ ConA.

Figure Legend

Figure 1

Various stages of Plasmodium inui in erythrocytes from a case of acute malaria in a cynomolgus monkey (Wright stain). Line = 6 μ m.

- A. Ring forms of very early trophozoites
- B. Trophozoite
- C. Developing schizont
- D. Mature schizont

