PLASMODIUM GALLINACEUM: CLINICAL PROGRESSION, RECOVERY, AND RESISTANCE TO DISEASE IN CHICKENS INFECTED VIA MOSQUITO BITE

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Abstract. Historically, in vivo experiments of Plasmodium gallinaceum in chickens have caused high mortality. Perhaps because of this high mortality, it remains to be demonstrated whether recovered birds will resist a second episode of illness when re-exposed to infected mosquitoes. In the current study, groups of 10 chicks were infected with P. gallinaceum via mosquito bite. Parasitemia and anemia were followed by recovery in all birds, although they had persisting, low levels of parasitized erythrocytes (0.007 ± 0.019%). Twenty-three days after the initial exposure, 10 recovered chicks were rechallenged with P. gallinaceum via mosquito bite; none of them developed clinical or hematological evidence of malaria, in contrast to matched control birds, which all became diseased ($P < 0.001$). Unlike previous studies, the current experiment had no mortality in chickens infected with P. gallinaceum by mosquito bite. Recovered birds resisted disease from re-exposure to the same organism. The duration and nature of immunity or premunition remain to be determined.

INTRODUCTION

Investigations of avian hemoprotozoa have provided many fundamental discoveries about malaria. More than a century ago, male and female gametocytes were first identified in passerines infected with Haemoproteus columbae (a blood parasite closely related to the true avian malarias), and shortly thereafter sexual reproduction of this organism was elucidated.1 In 1898, Ronald Ross documented Plasmodium relictum sporozoites within both oocysts and salivary glands of Culex fatigans mosquitoes.2 Shortly thereafter, Ross was the first to prove transmission of Plasmodium by mosquitoes using P. relictum in sparrows.3 Remarkably, an early effective experimental immunization was developed in 1910 using avian malaria.4 In that experiment, Étiene Sergent demonstrated that canaries became refractory to mosquito-transmitted malaria after vaccination with attenuated sporozoites of P. relictum. Discovery of extra-erythrocytic stages of malaria was accomplished with an avian Plasmodium,5 and in vitro culture of blood stages was achieved well in advance of similar accomplishments with human plasmodia.6 More recently, Warburg and Miller7 achieved the first in vitro culture of the mosquito stage of any malaria parasite, using P. gallinaceum.

Plasmodium gallinaceum, which naturally infects jungle fowl and chickens, was reported by Émile Brumpt in 1935,8 based in part on the work of previous investigators. Shortly thereafter, Brumpt obtained a field isolate that he passaged through chickens and then shared with other laboratories.9 Several early investigators examined P. gallinaceum malaria in chickens after transmission by mosquitoes.9–12 Mortality was high in those studies, which may partially explain why those investigators did not test chicks after recovery from malaria, to see if they remained susceptible or had become refractory to reinfection with P. gallinaceum by mosquito transmission. The goals of the current study were to monitor the course of anemia in chickens having primary P. gallinaceum infections acquired from mosquitoes and to determine if recovered chickens would resist developing disease when re-exposed to infected mosquitoes.

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MATERIALS AND METHODS

Parasite. Plasmodium gallinaceum, strain 8A, was provided by the National Institutes of Health (courtesy R. Gwadz, L. Miller, and A. Laughinghouse). This strain was derived from the original isolate obtained seven decades ago by Émile Brumpt.9 In our laboratory, the organism was maintained by twice-weekly blood passage in hens with occasional transmission via mosquitoes.

Biological vector. Aedes aegypti mosquitoes were raised and maintained at 25°C, 77% relative humidity, with a 12:12 hour light:dark cycle. Mosquito cages were constructed of 454 mL cardboard food cups (Sweetheart Cup Co., Owings Mills, MD) with the lid modified to hold mesh over the top. Mosquitoes were fed 5% sucrose in water from moist cotton placed on the mesh top of the cages. Prior to blood feeding, the sucrose was removed for 10–12 hours.

For the experiments in Phase 1, 2-day to 4-day postemergence mosquitoes were fed blood meals from 11-day-old chickens for 20 minutes. Negative control mosquitoes fed from uninfected chickens, whereas mosquitoes that were to be infected fed from chickens with a rising parasitemia (between 5% and 70%). After blood feeding, mosquitoes were briefly immobilized by chilling (4°C), and approximately 100 blood-engorged mosquitoes were retained in each mosquito cage. Ten mosquitoes from representative cages were killed 6–8 days after blood feeding and used to confirm infection, using microscopic examination of midguts for the presence of oocysts; mosquito infection rates were ≥ 60%. Mosquitoes were used 10 days after blood feeding to bite new birds. Mosquitoes were observed while feeding and examined immediately afterwards; 80% to 100% of the mosquitoes in each cage were estimated to have engorged with blood. For Phase 2, mosquitoes were prepared and used as above, with the exception that mosquitoes were divided into paired cages after feeding from the same infected donor chick; one mosquito cage from each pair was later used to infect Group A (recovered birds) and the other was used at the same time to infect Group B (positive control birds).

Vertebrate host. Thirty female F1 hybrid New Hampshire x Synthetic Columbian chickens were obtained from the University of Illinois Poultry Farm. The chickens were maintained under a light:dark cycle of 12:12 hours, at 27°C. Chickens were fed a commercial chick feed (Purina nonmedicated Start
and Grow, Frankfort IN) and water ad libitum. To infect the birds, each was gently restrained in dorsal recumbency with wings extended, and a cage containing either infected or uninfected mosquitoes was inverted onto the bare ventral skin of one wing of each bird in experimental and negative control groups, respectively. After 15 minutes, mosquito cages were exchanged between birds within the same group and applied to the opposite wing for another 15 minutes. This use of experimental animals was approved by the University of Illinois’ Institutional Animal Care and Use Committee, which ensures compliance with United States Department of Agriculture and National Institutes of Health guidelines for the humane use of laboratory animals.

Phase 1. At 23 days of age, 30 chickens weighing between 373 and 463 g were randomly divided into three groups of 10 birds each. Group A was infected via mosquito bite with *P. gallinaceum*. Group B was the negative control; these birds were sham-infected by the bite of uninfected mosquitoes. Group C was held in reserve until Phase 2. Each day at 8:00 AM, approximately 100 µL of blood from a wing vein of each bird was collected into a hematocrit tube. A small drop of this blood was smeared on a glass slide, stained using modified Giemsa (Sigma Diagnostics, St. Louis, MO), and examined microscopically for percent parasitemia (i.e., % parasitized erythrocytes) and polychromatophil counts (polychromatophils are the avian equivalent of mammalian reticulocytes, which increase in regenerative anemias). The hematocrit tube was centrifuged for measurement of packed cell volume (PCV), using standard procedures.

Phase 2. After recovering from infection in Phase 1, Group A chickens were re-exposed to the bites of infected mosquitoes at 46 days of age (23 days after the Phase 1 exposure). At the same time, Group B chickens were bitten by infected mosquitoes for the first time, to serve as positive controls, and Group C chickens were bitten by uninfected mosquitoes, to serve as negative controls. At this time, the birds weighed between 890 and 1,201 g. As before, 100 µL blood samples were analyzed daily until parameters returned to normal. All birds were euthanized at the termination of the study and weighed. The spleens were removed, weighed, fixed in 10% neutral buffered formalin, and routinely processed for histopathology.

Statistics. Using the SAS Proc Mixed statistical program, a repeated measurement analysis ANOVA was used to compare percent parasitemia and PCV among groups. Tukey adjustments were made as needed. Splenic weights, expressed as a percentage of body weight, were compared among groups using Student’s *t* tests with a Bonferroni method adjustment to control for comparison among three groups, thereby establishing an accepted significance level of 0.0167 (i.e., 0.05/3).

RESULTS

All 10 Group A birds in Phase 1, exposed to infected mosquitoes at 23 days of age, and all 10 Group B birds in Phase 2, first exposed to infected mosquitoes at 46 days of age, developed clinical signs of illness, parasitemia, anemia, and increased polychromatophil counts (Figure 1). The earliest clinical sign was bright green coloration of feces for 2 days (due to increased hemoglobin catabolism and resulting increased biliverdin excretion). At the same time, the PCV began to drop, although parasitemia and polychromasia were not yet observed. Over the next 2–3 days, other clinical signs developed, including ruffled feathers, lethargy, and pallor of wattles and combs, which coincided with anemia. Parasitized erythrocytes were first observed 7 days after exposure to infected mosquitoes in all 10 Group A birds in Phase 1 but varied between 7 days (*N* = 5) and 8 days (*N* = 5) after exposure in Group B birds in Phase 2. All Group A chickens recovered from illness in Phase 1, and all Group B chickens recovered from illness in Phase 2. Hematological values in all chickens returned to normal, with the exception of persisting minimal parasitemia that was monitored daily in the 10 Group A chickens (0.014 ± 0.378 parasitized erythrocytes per 2,000, range = 0 to 2, mode = 0). Negative control chickens remained healthy, thus demonstrating that daily blood sampling had no effect upon hematological or clinical parameters.

Upon re-exposure of Group A (recovered) birds to infected mosquitoes in Phase 2, 0 of 10 chickens developed clinical signs of illness or alterations of hematological param-
eters (Figure 1); these results resembled those of the negative control group. At the same time, all positive control birds (Group B) developed clinical and hematological evidence of disease as noted above (Group A in Phase 2 differs from Group B in Phase 2: $P < 0.001$).

At the end of the experiment, birds and spleens were weighed, and spleens were examined histologically. Splenic sizes as a percentage of body weight ($\pm SE$) were $0.41 \pm 0.03$ in chronically infected Group A birds, $0.88 \pm 0.05$ in recently infected Group B birds, and $0.17 \pm 0.01$ in Group C negative control birds; these differences were statistically significant among all 3 groups ($P < 0.001$). Lymphoid hyperplasia was marked in recently infected birds and moderate in chronically infected birds. Brown pigment was scattered within splenic macrophages of infected birds, with a greater amount of pigment observed in chronically than in recently infected birds. Perl’s iron stain had no affinity for this pigment, which is therefore consistent with hematozoin.

Results of statistical comparisons are provided in Table 1.

**DISCUSSION**

The current study describes the course of *Plasmodium gallinaceum* infections in chickens that are infected by vector mosquitoes. After initial exposure to mosquitoes, all chickens developed parasitemia and anemia, followed by clinical recovery. Recovered birds were completely resistant to a second episode of disease after re-exposure to the organism via the bite of infected mosquitoes.

It may be questioned whether the 10 Group A chickens that recovered after primary exposure to malaria were in fact adequately challenged upon second exposure in Phase 2 of the experiment. However, the study design precludes an error of this nature. Ten positive control (Group B) chickens were bitten by identically prepared cages of mosquitoes at the same time, and all 10 developed malaria. If we conservatively estimate that the odds of receiving an adequate infectious challenge for each bird were only 0.50 (i.e., 10 of 20), then the probability would be less than $10^{-16}$ that all 10 birds in Group A could have received an inadequate challenge while all 10 birds in Group B could have simultaneously received an adequate challenge. Previous studies showed highly efficient infection of chickens after the bite of only 1 or 2 mosquitoes carrying *P. gallinaceum*; each chicken in the current experiment was bitten by at least 80 mosquitoes, and we estimate at least 48 of these should have carried the infection.

Avian malarias were studied extensively in the early 20th century. However, after the first rodent model of malaria was described in 1948, research of rodent plasmodia has become more popular than the study of avian plasmodia. Nevertheless, there are several compelling reasons why avian malarias should continue to be studied. Multiple studies have found that *Plasmodium gallinaceum* has a closer phylogenetic relationship to *P. falciparum*, compared with the malaria parasites of rodents. *Avian malarias* provide the opportunity to examine the interaction between a malarial parasite and its natural host, with naturally evolved immunity, instead of a model in an artificial host, such as *P. berghei* in mice or *P. falciparum* in *Aotus* monkeys. Chickens also make excellent research subjects because they are inexpensive and, in comparison to mice, provide abundant blood for study and culture of organisms. Because of their ease in cultivation, we used *Aedes aegypti* mosquitoes to infect chickens in the current experiment, but *P. gallinaceum* can infect mosquitoes from six genera, including anopheline mosquitoes that transmit *P. falciparum* in nature. This specific model, using 3- to 6-week-old birds infected via mosquito bite, is particularly attractive because there was 100% morbidity but no mortality.

Most previous studies of *P. gallinaceum* have infected chickens by blood passage. Such studies usually induced high mortality, unless birds were treated with antimicrobials or the organisms were attenuated. In young chickens, mortality rates may be decreased by reducing the size of the infectious challenge or by using older (and consequently larger) birds. Chickens that recover from blood-transmitted malaria become refractory to disease when reinfected with infected blood. Regarding mosquito-transmitted *P. gallinaceum*, prior experiments predominantly used hatchlings or chicks even younger than those in the current study. Thus, both the route of inoculation and the age of the birds appear to affect the ability of chickens to recover from *P. gallinaceum* infection, as mortality was high in the above studies but was nil in the current experiment.

The biology of sporozoite invasion of *P. falciparum* differs from *P. gallinaceum*, in that the former invades hepatocytes, whereas the latter invades macrophages. The development of sporozoites within the mosquito host, however, is nearly identical among all *Plasmodium* species. In fact, a protocol originally developed to produce sporozoites of *P. gallinaceum in vitro* has also been used with *P. falciparum* to produce sporozoites, thereby demonstrating similar growth requirements of the vector stages.

People in *P. falciparum*-endemic regions often show a chronic, low-grade parasitemia without apparent clinical symptoms (i.e., premunition). *Plasmodium gallinaceum* infections of chickens are similar in this respect, resulting in chronic, low-grade parasitemia with a lack of clinical signs. The size of the spleens increased markedly in chickens after infection, followed by a partial reduction of the splenomegaly. This finding is consistent with previous studies in chickens infected by blood transmission and resembles the situation in falciparum malaria of humans.

In summary, we found that chickens exposed to *Plasmodium gallinaceum* via mosquito bite became clinically ill and developed parasitemia and regenerative hemolytic anemia, which resolved without intervention or mortality. After re-
covery, birds continued to display a very low level of parasitism. When re-exposed to infected mosquitoes, recovered birds did not become anemic, have a resurgence of parasitism, or develop outward signs of disease. Acutely infected birds develop markedly enlarged spleens, which partially resolve with time. In comparison to malaria induced in chickens by inoculation of infected blood, malaria transmitted by mosquitoes has a longer prepatent period, lower peak parasitemia, lower mortality, and more closely approximates the naturally occurring disease.

Future studies to further define this intriguing animal model of falciparum malaria should include determination of the duration and character of immunity or premunition. It would be useful to investigate resistance of recovered chickens to challenge with a heterologous isolate of *P. gallinaceum*, but we are unaware of any available laboratory strains other than those descended from the original isolate provided 70 years ago by Brumpt.

Received June 15, 2005. Accepted for publication July 22, 2005.

Acknowledgment: The authors thank the University of Illinois’ Department of Veterinary Pathobiology for supporting this project.

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