INFECTION AND IMMUNITY, Apr. 2004, p. 2248–2253 Vol. 72, No. 4
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.4.2248–2253.2004

Measuring the Effects of an Ever-Changing Environment on Malaria Control

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Received 2 September 2003/Returned for modification 26 October 2003/Accepted 31 December 2003

The effectiveness of malaria control measures depends not only on the potency of the control measures themselves but also upon the influence of variables associated with the environment. Environmental variables have the capacity either to enhance or to impair the desired outcome. An optimal outcome in the field, which is ultimately the real goal of vaccine research, will result from prior knowledge of both the potency of the control measures and the role of environmental variables. Here we describe both the potential effectiveness of control measures and the problems associated with testing in an area of endemicity. We placed canaries with different immunologic backgrounds (e.g., naïve to malaria infection, vaccinated naïve, and immune) directly into an area where avian malaria, *Plasmodium relictum*, is endemic. In our study setting, canaries that are naïve to malaria infection routinely suffer approximately 50% mortality during their first period of exposure to the disease. In comparison, birds vaccinated and boosted with a DNA vaccine plasmid encoding the circumsporozoite protein of *P. relictum* exhibited a moderate degree of protection against natural infection ($P < 0.01$). In the second year we followed the fate of all surviving birds with no further manipulation. The vaccinated birds from the first year were no longer statistically distinguishable for protection against malaria from cages of naïve birds. During this period, 36% of vaccinated birds died of malaria. We postulate that the vaccine-induced protective immune responses prevented the acquisition of natural immunity similar to that concurrently acquired by birds in a neighboring cage. These results indicate that dominant environmental parameters associated with malaria deaths can be addressed before their application to a less malleable human system.

Avian malaria in the Baltimore, Md., area was first utilized for investigating environmental factors relating to the onset of malaria by Beier and colleagues (2, 3). Our study was initiated in an attempt to monitor the effect of variables that are not addressed in laboratory trials (e.g., vaccine effects on the acquisition of natural immunity, herd immunity, variation in the average mosquito bite rate in adjacent areas, and the effects of natural boosting on the vaccine response). To this end, we tested the effects of vaccination in a setting that allows for environmental flux similar to that associated with human malaria transmission in field settings. In this model, certain dominant parameters such as time, degree of physical separation between subjects, and the immunologic history of birds could be monitored and manipulated in place. Although one cannot assume that other variables remain constant, one can anticipate that changes in significant variables will ultimately be seen. In this context, we believe that natural *Plasmodium* infections in avian populations provide a unique setting for the study of host mortality and morbidity. The avian model yields information that would not be available during large-scale human trials: (i) an assessment of mortality rather than infection; (ii) a determination, with rigid criteria, of the cause of mortality; (iii) continued follow-up on vaccinated and nonvaccinated cohorts; and (iv) complete information on site and living conditions of the study population.

The need for another malaria model when several are already available requires some explanation. Much of the empirical scientific method is based on determinism, a belief that a controlled study with a fixed set of initial starting conditions will always unfold in a predictable manner. Unfortunately, because outcomes in many systems are chaotic in nature, the accurate prediction of outcome from input becomes difficult, if not impossible. Historically, the laboratory model for vaccine trials has involved reducing variables to a minimum number. By doing so, it is very possible that we have eliminated critical variables (e.g., the ecology of the local mosquitoes, the nature and number of carriers in the system, the inoculum dose, the degree of previous exposure to malaria, and the genetic background of vaccine recipients) that could influence an assessment of vaccine efficacy and further parasite transmission.

We chose to deliver avian malaria vaccine as DNA plasmid (15–17, 19). In mice and monkeys, DNA-based malaria vaccines, when delivered alone, have been demonstrated to induce both cellular- and antibody-dependent immune responses (1,

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Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
2004	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Measuring the Effects of an Ever-Changing Environment on Malaria Control	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Naval Submarine Medical Research Laboratory Naval Submarine Base New London Box 900 Bldg 148, Trout Avenue Groton, CT 06349-5900**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**16**	

7, 20, 22, 23), with a limited degree of protective immunity in mice (7, 23). However, using DNA vaccines was found to be an excellent method of priming the immune system for subsequent boosting by natural infection (21, 22, 29). We constructed a DNA plasmid encoding the circumsporozoite protein (CSP) of the avian malaria parasite *P. relictum* into a mammalian expression plasmid. We then tested the ability of this vaccine plasmid to protect canary populations at the Baltimore Zoo from malaria-related deaths.

MATERIALS AND METHODS

Study design. This study was a randomized, double-blind clinical trial conducted at the Baltimore Zoo (Druid Hill Park, Baltimore, Md.). Juvenile canaries that had been held indoors since hatching, had no previous exposure to avian malaria as determined by enzyme-linked immunosorbent assay (ELISA), and were at least 3 months old were selected for the study. Immunizations were performed at time point zero and at 4 weeks. Safety considerations included close monitoring for local inflammation and systemic symptoms after each immunization and throughout the trial. Canaries were released to the outdoor aviary 7 days after the second immunization.

In order to assess the baseline mortality rates of naturally infected, unvaccinated canaries at the study site, we performed a preliminary trial 1 year prior to the start of the vaccine trial. At the same site as the site for the vaccine trial, 27 canaries were placed in an outdoor aviary. The aviary was monitored daily for dead canaries or canaries showing signs of clinical disease.

Construction of DNA vaccine. The CSP gene was amplified from RNA collected from infected mosquitoes by using a Superscript II reverse transcription-PCR kit (Invitrogen) according to the manufacturer's instructions. Nucleotide residues encoding amino acids 1 to 379 of the CSP were amplified by PCR. All amplification reactions were performed with 0.5 U of AmpliTaq polymerase (Perkin Elmer), PCR buffer (50 mM potassium chloride, 10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$), 100 ng of template DNA, 20 pmol of each primer, and 200 M concentrations of each nucleotide (dATP, dCTP, dGTP, and dTTP) (Boehringer Mannheim). The amplification parameters were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 3 min for 30 cycles, with a final elongation step at 72°C for 7 min. The primers used to identify the CSP gene were sense 1101 (5′-ATGAAGAAATTAGCCATTTTATC-3′) and antisense 1069 (5′-ATAGCTAAACCTAACGAATTGC-3′), based on the published sequence of the *Plasmodium gallinaceum* CSP gene (27). This fragment encompasses the entire CSP coding sequence except the terminal nine amino acids. The cDNA of the *Plasmodium relictum* CSP was inserted into mammalian expression plasmid VR1020 as previously described (15, 16). Large cultures of *Escherichia coli* DH10B (Life Technologies) transformants were grown, and the plasmids were isolated with a pyrogen-free, plasmid maxi-prep kit (QIAGEN) according to the manufacturer's instructions. Six independent clones were sequenced to confirm the reading frame (data not shown).

Immunizations. The plasmid DNA was adjusted to a final concentration of 0.5 mg of DNA per ml in phosphate-buffered saline. Malaria-naïve canaries received two intramuscular injections of 0.05 ml, spaced 3 weeks apart, in the left pectoral muscle. Control birds received phosphate-buffered saline without the DNA vaccine.

Monitoring of canaries. Prior to vaccination, 10 μl of blood was taken from each canary for the ELISA as previously described (10). Once the birds were released outdoors, each aviary was monitored daily for dead canaries. Every dead canary was subjected to a full necropsy. Sections of spleen, liver, and kidney were fixed in 10% buffered formalin. Thin-film blood smears were prepared by using 7 to 10 μl of blood treated with heparin and stained with Diff-Quik (Baxter Healthcare Corp.) according to the manufacturer's instructions and examined under oil immersion by a Laborlux S light microscope (Bunton, Inc.). After the canaries returned to the indoor aviary, 10 μl of blood was collected and used for ELISAs.

Detection of *P. relictum* in *Culex* mosquitoes. RNA preparation, primer probe, and hybridization conditions were as described for avian malarias by McConkey et al. (26).

Statistical analyses. Statistical analyses of mortality rates were performed independently at two locations with consistent results. At the Naval Medical Research Institute, EPI6 was used to determine *P* values relating to vaccine efficacy, and confidence intervals were determined at the National Institutes of Health according to the methods of K. Rothman and S. Greenland (28a). Both calculations were also made by using the STATA program, version 6.0. (StataCorp LP, College Station, Tex.).

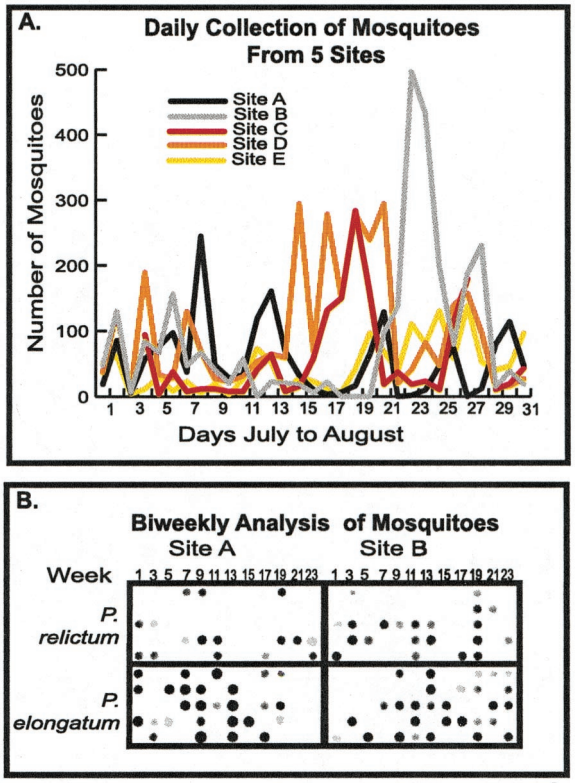

FIG. 1. Evaluation of mosquito infectivity in a defined area. (A) Time course showing the numbers of *Culex* mosquitoes collected at each of five different locations within a 1-km area. (B) Fifty mosquitoes collected from sites A and B from the first week in May to October were separated into batches of 10 each; RNA from each batch was spotted onto nitrocellulose filter paper. Each row of five dots represents RNA from the 50 mosquitoes collected on the indicated date. The filters were hybridized to oligonucleotide probes that detect only developing *P. relictum* sporozoites or only *P. elongatum* sporozoites (23, 25).

RESULTS

The *P. relictum* circumsporozoite gene. CSP genes were cloned from the RNA of mosquitoes infected with *P. relictum* and collected at the Baltimore Zoo. The identification of mosquitoes with only *P. relictum*, the most virulent avian malaria in the region, has been described (26). Twelve independent cDNA clones were sequenced and found to be identical to those found in *P. gallinaceum.* The result has been independently confirmed during a study of mosquito and bird infections in South Africa (K. C. Grim and T. F. McCutchan, unpublished data).

Incidence of *P. relictum*-related deaths in the canary population at Baltimore Zoo. Infection with avian *P. relictum* is highly virulent in previously nonexposed canaries. A mean survival rate was established in naïve canaries in 1999, prior to the vaccine trial (Fig. 1A). Thirteen of 27 canaries placed in an outdoor aviary died of malaria, indicating a probability of survival of approximately 50%. This rate is statistically indistin-

guishable from the death rate of unvaccinated canaries at location B in the year 2000 (Fig. 1) as well as consistent with the death rate among juvenile penguins at the Baltimore Zoo over a number of years (11). Similar to the results we show in Fig. 1, this number could vary to some extent; however, in our studies we have not found the rate to result in enough variation to obscure major trends.

Mosquito populations and *P. relictum* infection rate at the Baltimore Zoo. The importance of understanding the relationship between disease and transmission has been documented (13). Our study analyses were designed to detect significant swings in those factors that could influence trial outcome (e.g., the abundance of *Culex* mosquitoes, the percentage of infected mosquitoes, and variation in the parasite load of individual mosquitoes).

Mosquitoes were collected each morning over the spring and summer in Centers for Disease Control and Prevention gravid light traps at five different sites in the zoo compound. Collections from each night were counted, and the species were identified. The number of mosquitoes in a trap varied dramatically from day to day and between sites. Figure 1A shows collection data and shows that on a given day any one of the five sites (designated A through E) may represent the highest density of mosquitoes in terms of attraction to the light trap. Hence, mosquito density can shift dramatically within just a few days (30) even when traps are based on an identical attraction system. Two sites, A and B, had 6 days each when more than 100 *Culex* mosquitoes were collected. These sites were used for further analysis.

We initially looked for rapid and dramatic shifts occurring in the percentage of mosquitoes with developing sporozoites. Dramatic shifts in infection rate were determined by using a statistically based procedure described by Heymann and colleagues for the analysis of the frequency of chloroquine resistance infections (18). Two collection sites were sampled biweekly throughout the season. Fifty mosquitoes from each collection were divided into five batches of 10; RNA was then isolated from each batch and applied to nitrocellulose filters. The filters were hybridized with probes that could define both species and developmental progression (14, 24). The numbers of positive dots for a given date were used to determine whether a statistical distinction could be made between any two collections. For example, positive hybridization to either zero or one of the five dots has a definable probability of being different from a collection in which four of five dots test positively. A significant shift in the number of positive dots is shown in several situations (Fig. 1B), even when the samples were collected within 2 weeks of each other (e.g., *P. relictum* at site E during weeks 16 through 18). Dramatic differences of infectivity also occur between sites, as shown by the results in Fig. 1B.

The number of sporozoites inoculated during a mosquito bite is suggested to be a factor relating to disease (12, 13, 25). Based on the data available in this study, it was difficult to estimate the sporozoite inoculation levels with certainty. However, the different levels of hybridization to RNA certainly suggest that the number of developing sporozoite parasites varies (Fig. 1). Trends may thus emerge relating entomologic factors to mortality over the course of a number of trials.

Exposure of canaries to an open area of endemicity. Prior to vaccination, no birds used in the trial had been exposed to *P. relictum* sporozoites as determined by ELISA (data not shown). We selected the two most similar locations with regard to the average mosquito density, A and B. All canaries were put at either site A or B for 6 weeks during the trial. The 6-week exposure time was selected because it ensures that all birds will be challenged and, if infected, will develop malaria. We will investigate the effects of extending exposure in future experiments, and we emphasize that this may not be a generally appropriate time frame for other areas. In this location, however, we demonstrated that all birds were naturally challenged by sporozoites during that period as determined by ELISA (data not shown). Deaths were attributed to malaria when splenomegaly was apparent, malaria parasites were present in the peripheral blood, and there was no evidence of other pathogens. No further mortalities could be attributed to malaria after the canaries were returned to the indoor facility. Birds that died from other causes, including aspergillus infections, were eliminated from the study.

During the first year of exposure, 2 of 23 (8.6%) vaccinated canaries from site A died of malaria (Fig. 2B). During the same period, 16 of 31 (51.6%) nonvaccinated birds housed at site B (located 1 km from site A) died of malaria. The level of mortality at site B in 2000 and that at site A in 1999 were used as positive controls for the interpretation of protection levels in 2000. The data based on the described controls suggest that the DNA vaccine had a positive effect on the level of mortality ($P < 0.01$). We then asked whether vaccination influenced malaria-related mortality in nonvaccinated birds located in the same cage. By comparison, 4 of 27 (14.8%) nonvaccinated birds from site A died of malaria. If the malaria death rate in naïve populations is considered to be about 50%, we calculate that the vaccine also had an impact on malaria mortality in the nonvaccinated birds when they were housed together with the vaccine recipients. This result is neither unprecedented nor surprising if one assumes that herd immunity plays a role in the protection of nonvaccinated birds. Although the question arises as to whether herd immunity can have an effect within this amount of time, clearly it does. Virulent avian parasites usually are most life threatening within a short period after infection (9). It is known that in *Plasmodium elongatum* infection, exoerythrocytic forms pose the most serious threat (9), and birds die with little apparent blood-stage infection. We find this to be true with juvenile penguins at the Baltimore zoo as well. If we assume that canary-to-canary transmission is the major source of lethality, then the effects of lower levels of transmissibility would be seen very rapidly in the protection of neighbors.

Follow-up year. Birds surviving malaria exposure in the year 2000 were placed in a common environment (site A) during 2001. Site A, therefore, contained 8 vaccinated birds, 16 unvaccinated cage mates, and 12 unvaccinated control birds. Each group was banded so that the birds could be distinguished from each other at the end of 2001. When the banding code was broken, it was clear that the naturally immune birds could be distinguished on the basis of malaria mortality (12 alive and 0 dead), even when they had been housed with other birds (Fig. 2).

Curiously, all 12 birds that survived malaria exposure during

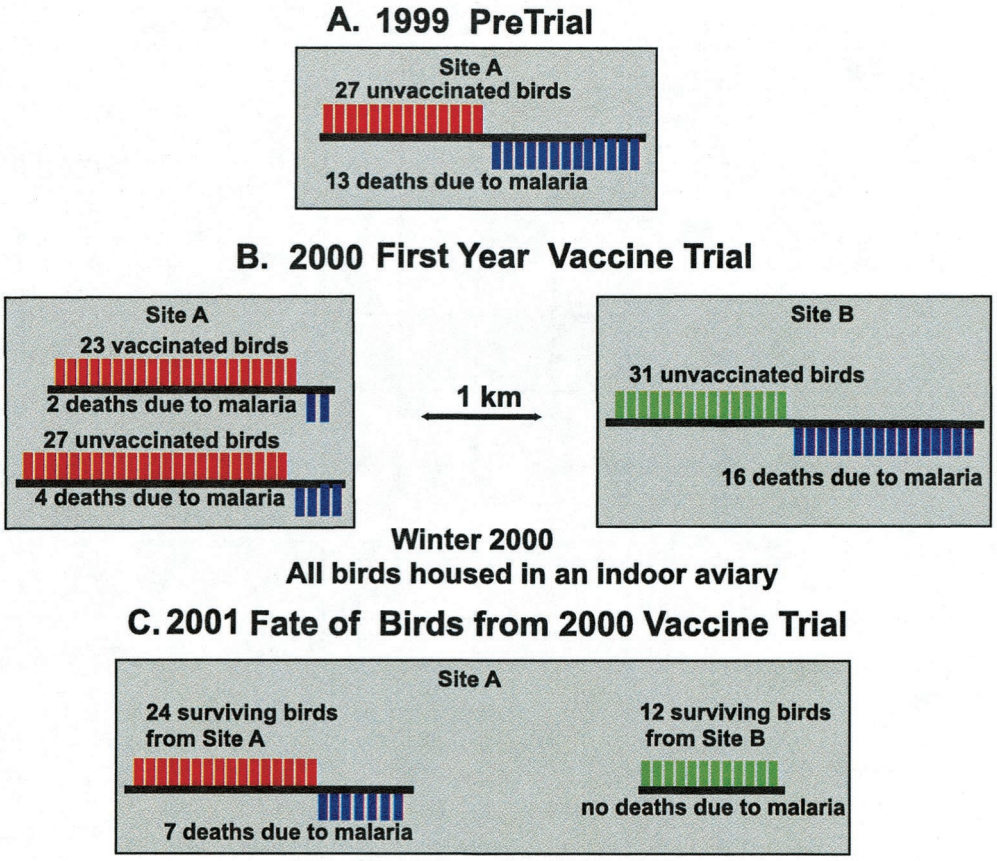

FIG. 2. The effect of an antimalarial vaccine on the mortality of canaries over a 3-year period. (A) Thirteen of 27 canaries put into an outside aviary in 1999 died of malaria (48% mortality). (B) Canaries were split randomly into two groups in 2000. One group was given a DNA vaccine, boosted, and mixed with unvaccinated canaries in an aviary at site A. The other group, housed at site B (~1 km away), suffered a mortality rate similar to that seen at site A the year before. (C) Survivors of the exposure in 2000 from both locations were put into site A during the 2001 season. All the birds previously from cage B (in 2000) survived this second exposure, while the birds from cage A (in 2000) suffered the mortality rates indicated in the figure.

2001 were the same ones that had been allowed to develop immunity in isolation at site B without vaccination. The canaries that had been relatively free of malaria in site A during 2000 responded very differently at that location in 2001. The vaccinated birds were more susceptible to death (3 of 8, $P = 0.05$), as were the nonvaccinated birds (4 of 16, $P = 0.07$), when compared with the 12 naturally immune birds described above. P values are difficult to assess accurately in this case, given that one factor is a zero. The results indicate a difference between the birds that had been vaccinated the prior year and the birds that developed natural immunity and were housed at the same location.

DISCUSSION

Determining the mechanisms of protection is the function, and the importance, of the laboratory trial. There are certain elements, however, relating to the success of a vaccine that are not addressed in the laboratory. Here, for the first time, we describe an in vivo challenge model for testing vaccine efficacy in a natural setting. There are distinct problems associated with testing in an open environment (e.g., determining what constitutes an appropriate control group), but they are the same problems that will need to be addressed when assessing a human vaccine in the field. Further, this model addresses the effect of a vaccine on (i) a nonvaccinated cohort living in close proximity to vaccinated individuals and (ii) the acquisition of reinfection immunity. We are cognizant of the fact that both the vaccine data and the information regarding the effect of herd immunity on vaccine outcome presented here are based on small sample sizes. An investigation of this type, however, should help us to develop an appropriate means of assessment of field trials for a human vaccine.

In this study, two effects of using a DNA vaccine in canary populations in the Baltimore Zoo were observed. The survival from death due to malaria was enhanced by the vaccine during the first year. Based on the results of ELISAs, all control birds were exposed to sporozoites during the trial, and hence the vaccine appeared to be responsible for protection. Also supporting this conclusion is the fact that nonvaccinated birds in site B died in significantly greater numbers than vaccinated birds in site A. The only consistent

difference between the sites of which we are aware is the vaccine.

Vaccination had another surprising effect. It seemed to interfere with the acquisition of reinfection immunity because in the follow-up year all birds that died were ones that had previously been vaccinated, while those that had acquired immunity as a result of exposure were fully protected. The relevance of these observations is discussed below.

DNA vaccines have been shown to be an excellent way to induce a class I-dependent cellular type of immune response. Because avian red cells are nucleated and express class I molecules, it is reasonable to assume that our DNA vaccine induced effective anti-CSP $CD8^+$-T-cell responses in vaccinated birds, leading to either a total elimination or significant reduction in blood-stage infection. Further, in murine malarias, DNA vaccine-induced immunity against preerythrocytic stage parasites is primarily mediated by class I-dependent $CD8^+$-T-cell responses (8).

We find that CSP is expressed in both preerythrocytic- and erythrocytic-stage parasites in *P. relictum* (K. C. Grim, J. Li, and T. F. McCutchan, unpublished data) and, therefore, anti-CSP immune responses could lead to either sterilizing immunity or a significant reduction in parasite burden. In the avian, there are other important differences in lymphatic organs such as the bursa of Fabricus. In this context, it is important to note that some of the most dramatic results from the use of DNA vaccines against infectious diseases have been achieved in avian models (28).

How the DNA vaccine was effective in protecting its neighbors remains unclear. Two factors that are known to be involved with malaria control are location effects and herd immunity. It is possible that a high degree of protection in nonvaccinated cage mates is the result of herd immunity. We speculate that vaccination-induced immune responses, both against sporozoites and blood-stage parasites, cause a significant reduction in parasite burden. With regard to avian malarias, this could be a factor in reducing inoculation rates within the time frame of the trial and lead to lower mortality rates. The effect of herd immunity on improved protection has been described in relationship to vaccine trials against Lyme disease (5). Herd immunity has been shown to play a critical role in the outcome of clinical trials of bed nets impregnated with insecticide in sub-Saharan Africa. The use of bed nets led to a significant reduction in child mortality in both the children that used them and their neighbors that did not (4, 6).

Location is also a factor in infection and could lead to an alternate interpretation of results. For example, there is a marked clustering of symptomatic malaria associated with particular sites, usually households (hence, references to a malaria house). Therefore, even though we have shown that the cage mates of the vaccinated individuals were challenged during the trial year, we acknowledge that symptomatic disease and exposure to sporozoites are different things. It is possible that symptomatic disease may not have occurred during the trial year in site A. This is possible despite the fact that normal mortality rates were seen there in both the preceding and following years.

The follow-up year indicated that even though every bird was challenged, the vaccinated birds and their cage mates had not acquired lasting immunity to disease in their first year of exposure. It appears that the acquisition of natural immunity was affected by the introduction of a vaccine that may have caused a total elimination or a significant reduction in parasite burden. This observation suggests that introduction of an effective vaccine in areas of endemicity could impair host immunity and worsen the malaria situation in the following transmission season. In conducting human trials, it would therefore be prudent to carefully monitor the trial subjects and their families and neighbors for an extended time.

As we point out above, there are alternative explanations of prevention data, as one might expect when subjects are introduced into a natural environment. Each alternative, however, suggests experiments to resolve the conflict with the avian model. No other model offers the opportunity to investigate these aspects of interplay between approaches to malaria prevention and the environment.

ACKNOWLEDGMENTS

We thank José Ribeiro for determining the probability associated with the data shown in Fig. 1 and 2 and Nancy Shulman for editorial advice and assistance in preparing the manuscript.

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Editor: W. A. Petri, Jr.