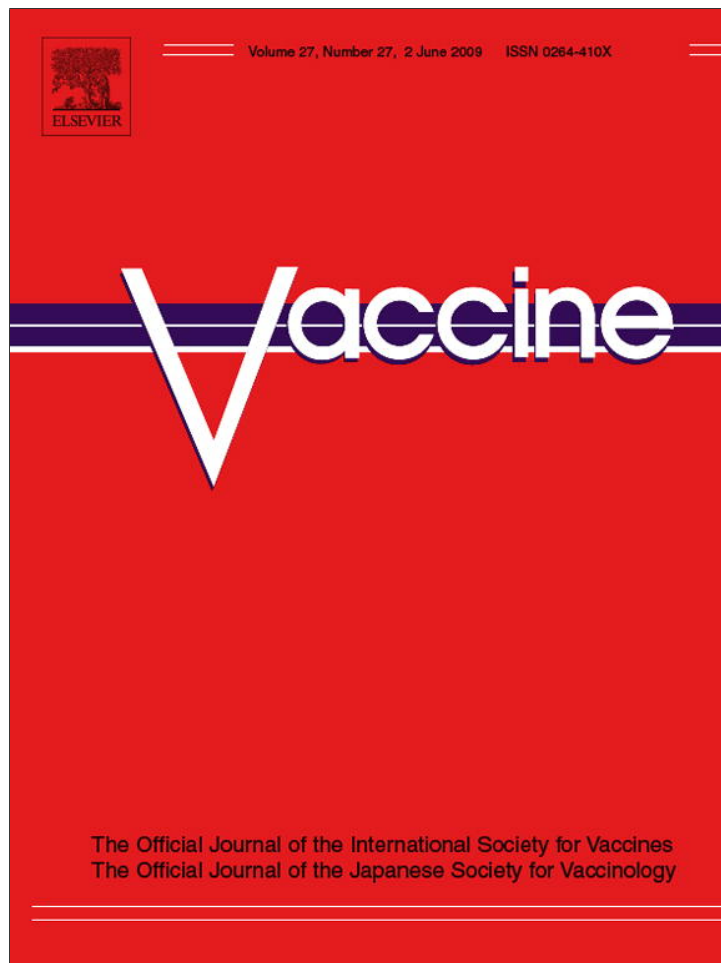


Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.



This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

<http://www.elsevier.com/copyright>



Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

The Effects of radiation on the safety and protective efficacy of an attenuated *Plasmodium yoelii* sporozoite malaria vaccine

Rana Chattopadhyay^{a,1}, Solomon Conteh^a, MingLin Li^{a,b}, Eric R. James^a,
Judith E. Epstein^{b,c}, Stephen L. Hoffman^{a,*}

^a Sanaria Inc., 9800 Medical Center Drive, Rockville, MD 20850, United States

^b Protein Potential LLC, 9800 Medical Center Drive, Rockville, MD 20850, United States

^c U.S. Military Malaria Vaccine Program, Naval Medical Research Center, Silver Spring, MD 20910, United States

ARTICLE INFO

Article history:

Received 9 September 2008

Received in revised form 5 November 2008

Accepted 17 November 2008

Available online 9 December 2008

Keywords:

Malaria

Vaccine

Sporozoites

Radiation

Attenuation

Plasmodium yoelii

ABSTRACT

We are developing a radiation attenuated *Plasmodium falciparum* sporozoite (PfSPZ) malaria vaccine. An important step was to determine the minimum dose of irradiation required to adequately attenuate each sporozoite. This was studied in the *Plasmodium yoelii* rodent model system. Exposure to 100 Gy completely attenuated *P. yoelii* sporozoites (PySPZ). Next we demonstrated that immunization of mice intravenously with 3 doses of 750 PySPZ that had received 200 Gy, double the radiation dose required for attenuation, resulted in 100% protection. These results support the contention that a radiation attenuated sporozoite vaccine for malaria will be safe and effective at a range of radiation doses.

© 2008 Elsevier Ltd. All rights reserved.

1. Introduction

We are developing a metabolically active, non-replicating *Plasmodium falciparum* sporozoite vaccine to prevent malaria [1,2]. The first generation vaccine will be attenuated by exposure to radiation. This is because there is a substantial body of data from human studies stretching back to the early 1970s demonstrating that sporozoites attenuated by radiation are potent inducers of protective immunity and that they are safe; they do not give rise to the asexual erythrocytic infections that cause malaria [1,3–8]. The sporozoites pass through the blood stream, invade hepatocytes, begin transformation to the next stage in the life cycle, liver stage trophozoites, express proteins not expressed in sporozoites, and then stop developing. Thus, they are metabolically active, but non-replicating.

Our approach to malaria vaccine development differs from that of most others in that we are developing a live attenuated (metabolically active, non-replicating) sporozoite vaccine, and essentially all other approaches to malaria vaccine development focus on subunit,

recombinant vaccines (http://www.who.int/vaccine_research/documents/en/Status.Table.pdf). A critical focus of our development process has been to determine the minimum dose of radiation that adequately attenuates all sporozoites, and thus ensures that the vaccine will not cause malaria. We are also interested in determining the highest dose of radiation that leaves the sporozoites able to elicit optimal protective immunity.

With *P. falciparum* sporozoites, experimental work can only be done *in vitro*, because there is no animal model of *P. falciparum* that faithfully reflects what occurs in humans. Small animals cannot be infected with *P. falciparum*. Several non-human primates, including *Aotus* sp. [9–11] and chimpanzees [12–14] can be infected with *P. falciparum*, but generally 10^4 – 10^5 sporozoites injected intravenously (IV) are required to achieve consistent asexual erythrocytic stage infection in 100% of recipients. In the case of *Aotus* sp. monkeys splenectomy is also required [11]. There are little quantitative data on administration by needle and syringe of *P. falciparum* sporozoites to humans, but in the case of *Plasmodium vivax*, as few as 10 sporozoites administered intradermally by needle and syringe have been shown to infect 100% of patients receiving malaria therapy [15]. It is presumed that *P. falciparum* sporozoites and *P. vivax* sporozoites have similar infectivity in humans. Results generated in a non-human primate model system that require 100–1000 times more *P. falciparum* sporozoites than in humans to achieve consistent infection cannot be expected to provide reliable information on

* Corresponding author. Tel.: +1 301 770 3222.

E-mail address: slhoffman@sanaria.com (S.L. Hoffman).

¹ Current address: Division of Emerging & Transfusion Transmitted Diseases, Office of Blood & Research Review, Center for Biologics & Evaluation, FDA, Bethesda, MD, United States.

the effects of different doses of irradiation on infectivity of sporozoites.

We use *in vitro* studies in the HC-04 human hepatocyte cell line [16] to assess the adequacy of attenuation and potency of our attenuated *P. falciparum* sporozoite vaccine. However, because the infectivity to mice of *Plasmodium yoelii* sporozoites (50% infectious dose [ID₅₀] <10 sporozoites) [17,18] is thought to be similar to the infectivity to humans of *P. falciparum* sporozoites, we have used the *P. yoelii* rodent model system to improve our understanding of the minimal doses of irradiation required to completely attenuate sporozoites, and the effects of increased doses of radiation on the capacity of the sporozoites to elicit a fully protective immune response.

2. Materials and methods

2.1. Mice

6–14-week-old female BALB/c mice (Harlan Laboratories, IN) were used in all but one experiment, in which CBySmn.CB17-Prkdc^{scid}/J BALB/c SCID mice were used.

2.2. Parasites

Non-lethal (clone 1.1) *P. yoelii* 17XNL [19] was maintained by alternating passage of the parasites in *Anopheles stephensi* mosquitoes and CD-1 mice (Harlan Laboratories, IN).

2.3. Mosquitoes

Female *A. stephensi* mosquitoes were used.

2.4. Infection of mosquitoes

5-day-old female *A. stephensi* mosquitoes that were ready for blood feeding were selected based on their migration to a heat source placed on one side of the container. Selected mosquitoes were subsequently fed on *P. yoelii* infected CD-1 mice and maintained at 24 °C for 14–17 days from the day of blood feed.

2.5. Irradiation of infected mosquitoes

Mosquitoes infected with *P. yoelii* sporozoites in containers were exposed to various radiation doses from a ⁶⁰Co source in batches depending on the experiment. The time of exposure of infected mosquitoes to achieve each of the target radiation doses was based on the calibration of the irradiator by dosimetry and the half life of ⁶⁰Co. The amount of radiation is reported in Gy [Gy = Gray, the International Standard (SI) unit of absorbed radiation dose].

2.6. Isolation and quantification of sporozoites

After irradiation, mosquito salivary glands of female *A. stephensi* mosquitoes were dissected from the mosquitoes into M-199 medium containing 10% normal mouse serum for 'immunization and challenge' experiments and M-199 containing 1% human serum albumin (HSA) for all other experiments. Sporozoites were then released from the salivary glands by passing the glands several times through a 1-ml syringe fitted with a 26½ G needle. The number of sporozoites was determined by counting in a hemocytometer.

2.7. Assessment of infectivity of PySPZ

To assess the infectivity of the *P. yoelii* sporozoites (PySPZ) used for the irradiation experiments, mosquitoes from the same contain-

ers were removed prior to irradiation and sporozoites were isolated. These non-irradiated PySPZ (3, 6, 12, or 24 PySPZs) were injected intravenously into groups of 5 mice to estimate the 50% infectious dose (ID₅₀) of the non-irradiated sporozoites. 7 and 14 days after inoculation of PySPZs, the tip of the mouse tail was pricked using a sterile lancet, a drop of blood was taken on a clean glass slide and a thin blood smear made. Slides were stained with 10% Giemsa in Milli-Q water, air dried, and assessed by microscopic examination of 50 high power (1000×) fields. A mouse was considered uninfected if the blood smears taken on days 7 and 14 were negative.

For each experiment, the % infected mice per group ($X/5 \times 100/1$) was plotted against the log₁₀ number of sporozoites +1, a linear regression was fit to the data and the regression equation used to calculate the number of sporozoites (antilog₁₀ -1 sporozoite) required to generate 50% infection (ID₅₀). The fit was best using log₁₀ transformed sporozoite doses that included a zero data point (an administration of 0 sporozoites would lead to 0% infection), and that excluded data points with 100% where the next lowest sporozoite dose also led to 100% infection (since above an ID₁₀₀ any additional sporozoites would also lead to 100% infection). These calculated ID₅₀ values are the results reported in the tables. We also estimated the ID₅₀ for all the experiments by calculating the mean of the estimates from individual experiments, with standard deviation (S.D.) estimated by the S.D. of the individual ID₅₀.

2.8. Assessment of infectivity of irradiated PySPZ

Groups of mice (generally 10/group) were injected intravenously with irradiated (50–100 Gy) PySPZ (1×10^5 PySPZ, except one experiment in which 1.54×10^5 irradiated PySPZs were used). Infectivity was assessed as above.

2.9. Assessment of the effect of increasing radiation dose on protective efficacy of radiation attenuated PySPZ

Mice were immunized by intravenous injection with PySPZ that had received either 100 Gy or 200 Gy. Two weeks after the last dose of irradiated PySPZ the mice were challenged by intravenous injection of 1000 non-irradiated PySPZ. Infection was assessed as above.

The animal experiments described in the study were approved (Animal Use Protocol # 07-01) by the IACUC committee of the Biomedical Research Institute (BRI), Rockville, MD. BRI's animal facility was used to house the mice and all experiments were conducted at BRI.

3. Results

3.1. Determination of the minimal dose of irradiation required to attenuate all PySPZ

We conducted a series of experiments with doses of radiation ranging from 50 Gy to 90 Gy. The data, which are outlined in Table 1, indicated that even 90 Gy did not attenuate all of the PySPZ, and that there was a highly significant correlation between the dose of irradiation and percentage of mice that developed parasitemia (Fig. 1, $r^2 = 0.98$). Based on the regression curve a dose of 92.24 Gy (95% confidence interval, lower CL = 86.53; upper CL = 101.26) was predicted to be required to achieve 100% attenuation of the sporozoites.

3.2. Lack of infectivity to BALB/c mice of PySPZ exposed to 100 Gy

In four different experiments, we injected a total of 41 mice each with 10^5 PySPZ that had been exposed to 100 Gy (Table 2). In no case was there a breakthrough asexual erythrocytic stage infection. In all

Table 1
Effect of increasing doses of irradiation on infectivity of *Plasmodium yoelii* sporozoites.

Radiation dose (Gy)	# Expt.	# Mice injected	ID ₅₀ of non-irr. PySPZ used	# Irr. PySPZ injected IV/mouse	# Mice parasitemic/# mice injected	Total # mice parasitemic/total # mice injected (%)
50	1	10	3.43	10 ⁵	10/10	10/10 (100)
60	1	6	3.37	1.54 × 10 ⁵	5/6	13/17 (76.4)
	2	11	1.97	10 ⁵	8/11	
70	1	10	1.97	10 ⁵	6/10	6/10 (60)
80	1	6	4.83	10 ⁵	0/6	3/14 (21.4)
	2	8	9.39	10 ⁵	3/8	
90	1	15	1.00	10 ⁵	2/15	2/25 (8)
	2	10	3.99	10 ⁵	0/10	

PySPZ-infected mosquitoes received 50–90 Gy from a ⁶⁰Co source, PySPZ were isolated from the mosquitoes, PySPZ were injected intravenously (IV) into BALB/c mice, and the mice were assessed for parasitemia on days 7 and 14 after infection.

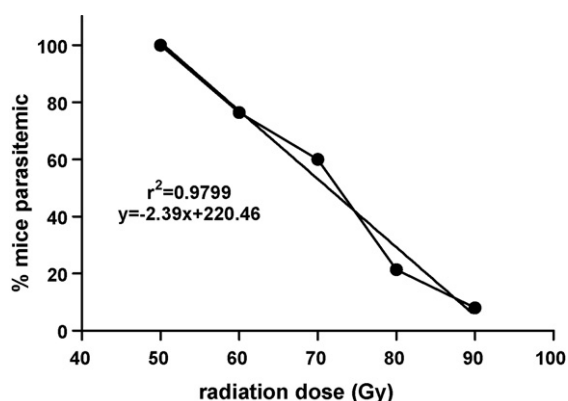


Fig. 1. Regression analysis of the effects of radiation dose on infectivity of sporozoites. The data in Table 1 were subjected to regression analysis. The analysis predicted that the minimum radiation dose needed to achieve 100% attenuation when 10⁵ PySPZ were injected IV into mice (x intercept for y=0) was 92.24 Gy (95% confidence interval, lower CL=86.53; upper CL=101.26).

three experiments all control BALB/c mice injected with 100 non-irradiated PySPZ developed an asexual erythrocytic stage infection. The ID₅₀ of non-irradiated PySPZ in these four independent experiments was 2.44, 2.39, 3.43 and 2.85 PySPZ, respectively (Table 2). The mice received a total of 4.1 × 10⁶ PySPZ, which was greater than 1.4 × 10⁶ ID₅₀s without a breakthrough infection.

3.3. Lack of infectivity to CBySmn.CB17-Prkdc^{scid}/J BALB/c SCID mice of PySPZ exposed to 100 Gy

Our understanding of radiation attenuation of *Plasmodium* sp. sporozoites is that the attenuation results entirely from physical events occurring in the radiation-exposed sporozoites. There is no evidence that in naïve recipients (never previously exposed to *Plasmodium* sp. parasites) host immune responses are required to achieve the attenuation. Nonetheless, we injected 10 severe combined B and T cell immunodeficient (SCID) CBySmn.CB17-Prkdc^{scid}/J

BALB/c mice with 10⁵ PySPZs that had been exposed to 100 Gy. None of these SCID mice developed an asexual erythrocytic stage parasitemia (Table 3). All 5 of the control SCID mice and 10 BALB/c mice that received 100 non-irradiated PySPZ from the same batch of mosquitoes developed an asexual erythrocytic stage infection. The ID₅₀ of the PySPZ used was 5.72.

3.4. Assessment of the effect of increased radiation dose on protective efficacy of PySPZ

Having established that 100 Gy is the minimum dose of radiation that attenuates all PySPZ, we wanted to begin the process of determining the maximum dose of irradiation that still provides protective immunity. *A. stephensi* mosquitoes infected with PySPZ are routinely exposed to 100 Gy to attenuate the sporozoites [20,21]. The literature indicates that exposing the infected mosquitoes to 200 Gy eliminates the protective efficacy of the sporozoites [22] even after a large immunizing dose. We therefore conducted an experiment comparing the protective efficacy of PySPZ that had received 100 Gy or 200 Gy. Because of the previous literature [22], we immunized BALB/c mice with high doses of sporozoites. Mice immunized with sporozoites that had received either 100 Gy or 200 Gy were equally protected (Table 4). Since we achieved 100% protection in both groups with high numbers of immunizing PySPZ (total of 5 × 10⁴–9 × 10⁴ irradiated PySPZ), we conducted a second experiment with the lower dose of PySPZ that we now routinely use (3 doses of 750 irradiated PySPZ, total of 2.250 × 10³ PySPZ). With this lower dosage regimen we achieved high levels of protection in the 100 Gy and 200 Gy groups (Table 4).

4. Discussion

We used the *P. yoelii* rodent model system to assess the effects of radiation on the infectivity and protective efficacy of *P. yoelii* sporozoites. The studies established that exposure to 100 Gy attenuated all sporozoites, and that immunization of mice with sporozoites exposed to 100 Gy or 200 Gy provided high level protective efficacy.

Table 2
Effect of 100 Gy on infectivity of *P. yoelii* sporozoites in immunocompetent BALB/c mice.

	# Expt.	# Mice injected	ID ₅₀ of non-irr. PySPZ used	# Irr. PySPZ injected IV/mouse	# Mice parasitemic/# mice injected (day 14)
BALB/c mice	1	6	2.44	10 ⁵	0/6
	2	10	2.39	10 ⁵	0/10
	3	10	3.43	10 ⁵	0/10
	4	15	2.85	10 ⁵	0/15
Total		41		4.1 × 10 ⁶	0/41

PySPZ-infected mosquitoes were exposed to 100 Gy from a ⁶⁰Co source, PySPZs were isolated from the mosquitoes, and mice were injected with 10⁵ PySPZ. None of 41 mice became infected indicating that a dose of 100 Gy attenuated all of the PySPZ.

Table 3
Effect of 100 Gy on infectivity of *P. yoelii* sporozoites in immunocompromised mice.

	# Mice	# Non-irr. PySPZ injected IV/mouse	# Irr. PySPZ injected IV/mouse	# Mice parasitemic/# mice injected (day 14)
CBySmn.CB17-Prkdc ^{scid} /J	10	0	10 ⁵	0/10
CBySmn.CB17-Prkdc ^{scid} /J	5	100	0	5/5
BALB/c	10	100	0	10/10

PySPZ-infected mosquitoes were exposed to 100 Gy from a ⁶⁰Co source, PySPZs were isolated from the mosquitoes, and CBySmn.CB17-Prkdc^{scid}/J mice were injected intravenously with 10⁵ PySPZs. None of 10 mice became infected indicating that a dose of 100 Gy attenuated all of the PySPZs. All control mice receiving 100 non-irradiated PySPZs from the same batch of mosquitoes became parasitemic. The ID₅₀ was 5.72.

We first established a highly significant ($r^2 = 0.98$, $p < 0.0001$) inverse association between the dose of irradiation and the infectivity of sporozoites from 50 Gy to 90 Gy (Table 1 and Fig. 1). A statistical model based on the data predicted that 92.24 Gy (lower and upper 95% confidence intervals, 86.53, 101.26) would achieve 100% attenuation. In this experiment, control groups of mice were injected in parallel with non-irradiated *P. yoelii* sporozoites, and the 50% infectious dose (ID₅₀) of non-irradiated sporozoites from the same mosquitoes averaged 3.68 ± 2.61 . We therefore conducted 4 different experiments in which we injected at least 10⁵ *P. yoelii* sporozoites that had been exposed to 100 Gy into each of 41 different mice and found that none of the mice became infected (Table 2). There is no evidence that host immunity has any influence on the attenuation of sporozoites by radiation. Nonetheless, we repeated the experiments with sporozoites exposed to 100 Gy in immunodeficient CBySmn.CB17-Prkdc^{scid}/J BALB/c SCID mice. Again there were no breakthroughs (Table 3). We interpret these results to indicate that once a threshold level of irradiation has been reached all sporozoites are attenuated sufficiently to prevent infection of mice. With *P. yoelii* sporozoites in BALB/c mice the threshold was between 90 Gy and 100 Gy.

We next established that immunization of mice with three doses of only 750 *P. yoelii* sporozoites (total dosage of 2250 sporozoites) exposed to either 100 Gy or 200 Gy provided high level protection. 100% of 10 mice immunized with sporozoites subjected to 200 Gy were protected. These data demonstrated that one can immunize with sporozoites exposed to significantly more irradiation than the minimum required dose, and still achieve complete protection. These findings were not consistent with those reported by Mellouk et al. [22], who found that immunization with two doses (7×10^5 and 3×10^5 , total 1×10^6) of *P. yoelii* sporozoites exposed to 100 Gy achieved 100% protection, and administration of the same dosage regimen of PySPZ exposed to 200 Gy gave no protection. Mellouk's studies [22] differed from ours in several ways. First, almost three orders of magnitude more PySPZ were administered. It has been suggested that beyond a threshold, increasing the dose of irradiated PySPZ is associated with decreased protective efficacy (Martha Sedegah, personal communication). Second, in our studies sporozoites used for immunization were isolated from hand-dissected

salivary glands. Those in Mellouk's study were unlikely to have been isolated from hand-dissected salivary glands. It is more likely that they were separated from mosquitoes by centrifugation using the Ozaki technique [23] (Martha Sedegah, personal communication). Our data indicate that there is a 500-fold reduction in infectivity of sporozoites isolated by density gradient centrifugation as compared to isolation by dissection (unpublished), and it is possible that a similar reduction in infectivity of sporozoites occurs with the Ozaki technique. Third, we irradiated the PySPZ by exposing the mosquitoes carrying sporozoites to irradiation, not by irradiating the sporozoites directly. One cannot tell from the paper, but it is possible that Mellouk irradiated the sporozoites after isolation from the salivary glands. In our case, mosquito tissues could have absorbed some of the radiation, and the dose of irradiation received by the sporozoites could have been lower than received by sporozoites irradiated directly. Fourth, and probably most important, Mellouk et al. immunized with two doses of irradiated sporozoites, and we used three doses. In our experience two IV doses of irradiated sporozoites rarely provides solid protection, while three IV doses always provides solid protection (unpublished). A profound difference in the protective efficacy of two vs. three doses of irradiated sporozoites was also recently reported in another study [24]. Thus, there are a number of possible explanations for the differences between these results. Regardless, the murine system is only a model. The optimal route and method of administration of the PfSPZ vaccine attenuated by exposure to a defined dose of irradiation will be established in human clinical trials. Once these parameters have been established, it will be determined, if it is advisable to assess the effect of increasing the dose of irradiation on protective efficacy in humans.

Many live attenuated vaccines are contraindicated or used with caution in individuals who are immunocompromised. This is because the host's immune system plays a role in protecting immunocompetent individuals from dissemination of the attenuated vaccine, and there is an increased chance of dissemination in immunocompromised individuals. We have conducted many studies *in vitro* in the HC-04 human hepatocyte cell line with irradiated *P. falciparum* sporozoites. The irradiated *P. falciparum* sporozoites invade these cells and produce a protein that was not expressed

Table 4
Effect of 100 Gy or 200 Gy on protective efficacy of *P. yoelii* sporozoites.

# Expt.	Group	# Mice	Radiation dose (Gy)	Immunizing dose of PySPZ ($\times 10^{-3}$)	Challenge dose of PySPZ	# Protected/# challenged	Protection (%)
1	1	6	100	70/10/10	10 ³	6/6	100
	2	6	200	30/10/10	10 ³	6/6	100
	3	6	100	70/10/10	10 ³	6/6	100
	4	6	200	30/10/10	10 ³	6/6	100
2	1	10	100	0.75/0.75/0.75	10 ³	9/10	90
	2	9	200	0.75/0.75/0.75	10 ³	9/9	100

PySPZ-infected mosquitoes were irradiated with either 100 Gy or 200 Gy from a ⁶⁰Co source. PySPZs were isolated from the mosquitoes and mice were immunized by the IV route 3 times at 2-week intervals using as many as 70×10^3 irradiated PySPZ and as few as 0.75×10^3 irradiated PySPZ per dose, and challenged 2 weeks after the last immunization by IV injection of 10³ PySPZ. Immunization with 3 doses of 0.75×10^3 irradiated PySPZ gave consistently high protection. Immunization with PySPZ that received 100 Gy or 200 Gy of irradiation led to the same degree of protection. In experiment #1 the ID₅₀ of the sporozoites used for challenge was 5.72 sporozoites and in experiment #2 the ID₅₀ of the sporozoites used for challenge was 6.67 sporozoites.

in sporozoites (indicating that they are metabolically active), but they never mature to the late liver stage (non-replicating). These studies demonstrate that in HC-04 human hepatocyte cells, in the absence of cells that can contribute to innate or adaptive immunity such as Kupffer cells, T cells, NK cells, and dendritic cells, irradiation adequately attenuates the sporozoites rendering them non-replicating. The studies reported herein conducted with immunocompromised SCID mice (Table 3) support the hypothesis that radiation attenuated PfSPZ will be safe if administered as a vaccine to immunocompromised individuals.

In 1989 we began immunizing volunteers by the bite of *P. falciparum*-infected *A. stephensi* mosquitoes that had been exposed to 15 krad (= 150 Gy) from a ^{60}Co or ^{137}Cs source [25]. The goal of the project was to study the immune mechanisms and antigenic targets of the protective immunity [1,2]. In these studies and studies conducted at the University of Maryland [26] during the same time period, 14 volunteers were each immunized by the bite of greater than a thousand *P. falciparum* sporozoites irradiated with ≥ 15 krad (= 150 Gy), and there were no breakthrough infections [1].

In 1989 we chose the radiation dose of 15 krad (= 150 Gy) based on studies that had been performed in the early 1970s. Two groups had demonstrated that irradiation of mosquitoes infected with *P. falciparum* or *P. vivax* sporozoites with >12 krad (= 120 Gy) from an X-ray machine prevented breakthrough infections, while irradiation with ≤ 12 krad (= 120 Gy) resulted in a significant number of breakthroughs. Between 1971 and 1975, in studies conducted by Rieckmann et al. [4,6], the target irradiating dose was 12 krad (minimum of 12 krad or 120 Gy); two of eleven volunteers in these studies had breakthrough infections after immunization. In studies conducted by Clyde et al. [3,5,7], volunteers immunized with sporozoites irradiated with a mean dosage of 17.5 krad (= 175 Gy) had no breakthroughs, while 4 of 7 volunteers immunized by sporozoites irradiated with 12 krad (= 120 Gy) developed breakthrough infections. No volunteer immunized with *P. falciparum*-infected mosquitoes that received 150 Gy or more has ever had a breakthrough *P. falciparum* infection. However, one individual in the University of Maryland studies (DFC), who had a complicated history of immunizations and challenges, developed *P. vivax* malaria after immunization with *P. vivax* Chesson strain sporozoites irradiated with 15 krad (= 150 Gy). However, prior to immunization, the volunteer had been exposed to non-attenuated *P. vivax* Chesson strain sporozoites and had become parasitemic. Thus, Clyde noted the development of infection after immunization may have been a coincidental relapse from hypnozoites from the earlier *P. vivax* infection.

The existing human data provide firm evidence that an irradiating dose to mosquitoes of ≥ 150 Gy is adequate to render *P. falciparum* sporozoites, and probably *P. vivax* sporozoites, administered by the bite of mosquitoes incapable of replication within the human host. The data we have generated in mice with *P. yoelii* sporozoites indicate that once a threshold level of irradiation has been achieved there will never be breakthrough infections. In *P. yoelii* 100 Gy is definitely above that threshold. Thus, the murine data with *P. yoelii* suggest that 150 Gy is above the threshold for radiation attenuation for *P. falciparum* sporozoites administered to humans, and if *P. falciparum* sporozoites are exposed to ≥ 150 Gy, the vaccine will be safe. This is an excellent starting point for initial safety studies in clinical trials. However, it will take extensive testing in humans to prove that exposure of *P. falciparum* sporozoite-infected mosquitoes to a specific level of radiation produces adequate attenuation of all *P. falciparum* sporozoites administered as a vaccine. The data in mice showing that immunization with *P. yoelii* sporozoites exposed to double the minimal dose required for attenuation (200 Gy) provides 100% protection are important. They suggest that once a threshold minimum

safe dose of irradiation to *P. falciparum* sporozoites has been established in humans, and the vaccine has been shown to be protective at that dose of radiation, it should be possible to raise that dose significantly above the minimal threshold, thereby increasing the safety profile of the vaccine without reducing the protective efficacy of the vaccine.

Acknowledgements

We thank Dr. Martha Sedegah, U. S. Military Malaria Vaccine Program, Naval Medical Research Center/Walter Reed Army Institute of Research, Silver Spring, MD for advice. We also thank Steve Matheny and the rest of the Sanaria Mosquito Production team for providing mosquitoes, Aderonke Awe for dissection of mosquitoes, LiXin Gao for processing of sporozoites, and Dr. Charles Anderson for editing. Dr. Judith E. Epstein is a military service member; her contribution to the manuscript was conducted as part of her official duties. The opinions and assertions herein are the private ones of the authors and are not to be construed as official or as reflecting the views of the US Navy or the Department of Defense. This work was supported by SBIR Phase I grant 1R43AI058499-01 from NIAID/NIH, grant Sanaria-01 from The Institute of One World Health and grant GAT0005-07276-COA.A from the PATH-Malaria Vaccine Initiative.

References

- [1] Hoffman SL, Goh LM, Luke TC, Schneider I, Le TP, Doolan DL, et al. Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium falciparum* sporozoites. *J Infect Dis* 2002;185:1155–64.
- [2] Luke TC, Hoffman SL. Rationale and plans for developing a non-replicating, metabolically active, radiation-attenuated *Plasmodium falciparum* sporozoite vaccine. *J Exp Biol* 2003;206:3803–8.
- [3] Clyde DF, Most H, McCarthy VC, Vanderberg JP. Immunization of man against sporozoite-induced falciparum malaria. *Am J Med Sci* 1973;266:169–77.
- [4] Rieckmann KH, Carson PE, Beaudoin RL, Cassells JS, Sell KW. Letter: sporozoite induced immunity in man against an Ethiopian strain of *Plasmodium falciparum*. *Trans R Soc Trop Med Hyg* 1974;68:258–9.
- [5] Clyde DF. Immunization of man against falciparum and vivax malaria by use of attenuated sporozoites. *Am J Trop Med Hyg* 1975;24:397–401.
- [6] Rieckmann KH, Beaudoin RL, Cassells JS, Sell KW. Use of attenuated sporozoites in the immunization of human volunteers against falciparum malaria. *Bull World Health Organ* 1979;57(Suppl. 1):261–5.
- [7] Clyde DF. Immunity to falciparum and vivax malaria induced by irradiated sporozoites: a review of the University of Maryland studies, 1971–75. *Bull World Health Organ* 1990;68(Suppl.):9–12.
- [8] Rieckmann KH. Human immunization with attenuated sporozoites. *Bull World Health Organ* 1990;68(Suppl.):13–6.
- [9] Collins WE, Skinner JC, Broderson JR, Huong AY, Mehaffey PC, Stanfill PS, et al. Infection of *Aotus azarae* boliviensis monkeys with different strains of *Plasmodium falciparum*. *J Parasitol* 1986;72:525–30.
- [10] Collins WE, Galland GG, Sullivan JS, Morris CL, Richardson BB, Roberts JM, et al. The Santa Lucia strain of *Plasmodium falciparum* as a model for vaccine studies. I. Development in *Aotus lemurinus* griseimembra monkeys. *Am J Trop Med Hyg* 1996;54:372–9.
- [11] Gramzinski RA, Obaldia 3rd N, Jones TR, Rossan RN, Collins WE, Garrett DO, et al. Susceptibility of Panamanian *Aotus lemurinus lemurinus* to sporozoite-induced *Plasmodium falciparum* (Santa Lucia) infection. *Am J Trop Med Hyg* 1999;61:19–25.
- [12] Walliker D, Quakyi IA, Wellems TE, McCutchan TF, Szarfman A, London WT, et al. Genetic analysis of the human malaria parasite *Plasmodium falciparum*. *Science* 1987;236:1661–6.
- [13] Meis JF, Ponnudurai T, Mons B, van Belkum A, van Eerd PM, Druilhe P, et al. *Plasmodium falciparum*: studies on mature exoerythrocytic forms in the liver of the chimpanzee, Pan troglodytes. *Exp Parasitol* 1990;70:1–11.
- [14] Daubersies P, Thomas AW, Millet P, Brahimi K, Langermans JA, Ollomo B, et al. Protection against *Plasmodium falciparum* malaria in chimpanzees by immunization with the conserved pre-erythrocytic liver-stage antigen 3. *Nat Med* 2000;6:1258–63.
- [15] Ungureanu E, Killick-Kendrick R, Garnham PC, Branzei P, Romanescu C, Shute PG. Prepatent periods of a tropical strain of *Plasmodium vivax* after inoculations of tenfold dilutions of sporozoites. *Trans R Soc Trop Med Hyg* 1976;70:482–4.
- [16] Sattabongkot JN, Yimamnuaychoke S, Leelaudomlapi S, Rasameesoraj M, Jenwithisuk, Coleman RE, et al. Establishment of a human hepatocyte line that supports in vitro development of the exo-erythrocytic stages of the malaria parasites *Plasmodium falciparum* and *P. vivax*. *Am J Trop Med Hyg*; 74: 708–15.

- [17] Khusmith S, Charoenvit Y, Kumar S, Sedegah M, Beaudoin RL, Hoffman SL. Protection against malaria by vaccination with sporozoite surface protein 2 plus CS protein. *Science* 1991;252:715–8.
- [18] Khusmith S, Sedegah M, Hoffman SL. Complete protection against *Plasmodium yoelii* by adoptive transfer of a CD8+ cytotoxic T-cell clone recognizing sporozoite surface protein 2. *Infect Immun* 1994;62:2979–83.
- [19] Weiss WR, Good MF, Hollingdale MR, Miller LH, Berzofsky JA. Genetic control of immunity to *Plasmodium yoelii* sporozoites. *J Immunol* 1989;143:4263–6.
- [20] Doolan DL, Hoffman SL. IL-12 and NK cells are required for antigen-specific adaptive immunity against malaria initiated by CD8+ T cells in the *Plasmodium yoelii* model. *J Immunol* 1999;163:884–92.
- [21] Sedegah M, Weiss WW, Hoffman SL. Cross-protection between attenuated *Plasmodium berghei* and *P. yoelii* sporozoites. *Parasite Immunol* 2007;29:559–65.
- [22] Mellouk S, Lunel F, Sedegah M, Beaudoin RL, Druilhe P. Protection against malaria induced by irradiated sporozoites. *Lancet* 1990;335:721.
- [23] Ozaki LS, Gwadz RW, Godson GN. Simple centrifugation method for rapid separation of sporozoites from mosquitoes. *J Parasitol* 1984;70:831–3.
- [24] Kumar KA, Sano G, Boscardin S, Nussenzweig RS, Nussenzweig MC, Zavala F, et al. The circumsporozoite protein is an immunodominant protective antigen in irradiated sporozoites. *Nature* 2006;444:937–40.
- [25] Egan JE, Hoffman SL, Haynes JD, Sadoff JC, Schneider I, Grau GE, et al. Humoral immune responses in volunteers immunized with irradiated *Plasmodium falciparum* sporozoites. *Am J Trop Med Hyg* 1993;49:166–73.
- [26] Herrington D, Davis J, Nardin E, Beier M, Cortese J, Eddy H, et al. Successful immunization of humans with irradiated malaria sporozoites: humoral and cellular responses of the protected individuals. *Am J Trop Med Hyg* 1991;45:539–47.