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Maintenance of protective immunity against malaria by persistent hepatic parasites derived from irradiated sporozoites

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ABSTRACT **Immunization of rodents and humans with irradiation-attenuated malaria sporozoites confers preerythrocytic stage-specific protective immunity to challenge infection. This immunity is directed against intrahepatic parasites and involves T cells and interferon γ, which prevent development of exoerythrocytic stages and subsequent blood infection. The present study was undertaken to determine how protective immunity is achieved after immunization of rodent hosts with irradiated *Plasmodium berghei* sporozoites. We present evidence that irradiated parasites persist in hepatocytes of rats and mice for up to 6 months after immunization. A relationship between the persistence of parasites and the maintenance of protective immunity was observed. Protective immunity was abrogated in irradiated-sporozoite-immunized rats following the application of chemotherapy to remove preexisting liver parasites. Additionally, protective immunity against sporozoite challenge was established in rats vaccinated with early and late hepatic stages of irradiated parasites. These results show that irradiation-attenuated sporozoites produce persistent intrahepatic stages *in vivo* necessary for the induction and maintenance of protective immunity.**

The potential of using irradiation-attenuated parasites to develop an effective malaria vaccine has been recognized since 1941, when Mulligan *et al.* (1) demonstrated that immunization of chickens with x-irradiated *Plasmodium gallinaceum* sporozoites (spz) induced protective immunity. Since then, vaccination studies using x- or γ-irradiated rodent or human malaria sporozoites (γ-spz) have consistently resulted in a high level of protection against a live homologous spz challenge (2). In contrast, immunization of human or other vertebrate hosts with chemically treated, killed spz or even some synthetic or recombinant forms of defined antigens from this stage of the parasite has not achieved the high levels or duration of protection that result from vaccination with γ-spz (3–6). Unfortunately, large-scale immunization of humans with γ-spz is not practical due to the difficulties of producing infective spz *in vitro* and the ethical considerations involved in immunizing large numbers of individuals by the bite of infected mosquitoes. Understanding the mechanism by which such a potent protective immunity is induced by γ-spz could lead to the discovery of important liver stage antigens with the potential for subunit malaria vaccine development.

The invasion of host hepatocytes by γ-spz, their transformation into liver stages, their development within the infected hepatocyte, and the development of either a blood stage infection or protection from spz challenge are dependent upon the irradiation dose administered to the spz (7, 8). The generation of this protective immunity requires the delivery of live γ-spz to the host, γ-spz invasion into hepatocytes, and γ-spz transformation and further development to exoerythrocytic stages (EEs) (2, 9). The γ-spz-induced protective immunity has previously been shown to involve interferon γ and $CD8^+$ T cells directed against liver stage parasites, and *in vivo* depletion of these effectors has initiated blood infection (10). To determine those processes involved in stimulation of the host protective immune responses and the steps necessary for the maintenance of long-lasting protective immunity, we have investigated the EEs of the γ-spz (EErads) by using the *Plasmodium berghei*–rat model. These results demonstrated that EErads can persist for up to 6 months after immunization of mice with γ-spz, thus confirming the finding of Suhrbier *et al.* (8) that EErads persist in Hep G2 cells *in vitro* for up to 10 days. In addition, protective immunity was abrogated in γ-spz-immunized rats following the application of chemotherapy to remove preexisting liver parasites.

MATERIALS AND METHODS

Animals. The animals used in this study were cared for and used strictly in accordance with the Public Health Service guidelines (11).

Immunization. *Anopheles stephensi* mosquitoes infected with ANKA strain of *P. berghei* were irradiated with 10 krad (1 rad = 0.01 Gy) from a ^{137}Cs source. The isolation of spz from mosquitoes was performed on a biphasic Renografin gradient (12). Rats were immunized with 3×10^6 γ-spz by the hepatic portal branch inoculation method, as described (13, 14). Controls consisted of rats immunized with mosquito salivary gland lysates.

Liver Resections and Detection of EErads. Rats that received either spz or γ-spz via hepatic portal branch inoculation were killed at various times after immunization, and their livers were removed for DNA *in situ* hybridization and immunological assays. Liver samples were embedded in Tissue-Tek O.C.T. compound (Miles), quick frozen in liquid nitrogen-chilled isopentane, and stored at −80°C until sectioned. Serial frozen sections (5 μm) were cut in a cryostat, fixed with chilled methanol before being processed for immunofluorescence assays (IFA), and fixed with 4% formaldehyde buffer before being processed for DNA *in situ* hybridization. The DNA *in situ* hybridization with a digoxigenin-labeled probe specific for the subtelomeric repeat region of DNA of *P. berghei* (15) was performed on liver sections as described (16). Parallel IFA and Giemsa-staining were also carried out to confirm the presence of liver-stage parasites. The mean number of EEs or EErads per cm^2 was determined for each group at different time points after immunization.

Treatment with Primaquine. To investigate the role of persistent EErads in protecting γ-spz-immunized animals from subsequent spz challenge, primaquine was used to eliminate EErads. At 7 days postimmunization, rats were treated with phosphate-buffered saline (PBS; group I, see Table 1) or with primaquine salt (groups II and III; see Table 1). Treatment consisted of two doses, 10 h apart, of 60 mg of the primaquine salt (WR002975) per kg injected subcutaneously

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Abbreviations: spz, sporozoites; γ-spz, γ-irradiated spz; EEs, exoerythrocytic stages; EErads, EEs of the γ-spz.

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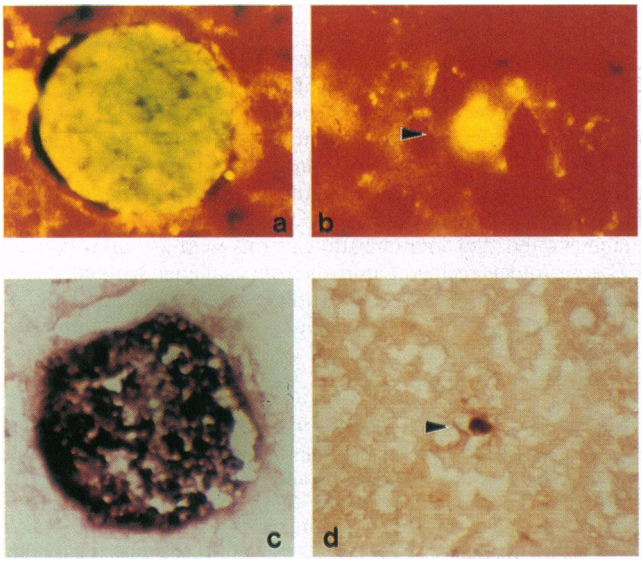

FIG. 1. EEs of *P. berghei* in rat liver sections. (*a* and *b*) Immunofluorescence assay. (*a*) EEs (46-h; 40 μm) stained with sera from rats immunized with blood stages of *P. berghei*. (*b*) EErads (46-h; 5 μm) (arrowhead) stained with pooled sera from rats immunized with 10-day EErads (1:50 dilution). (Monoclonal antibodies to circumsporozoite protein and sera from rats immunized with blood stages could not be used because of their poor reactivity with the >46-h EErads.) (*c* and *d*) DNA *in situ* hybridization of 44-h *P. berghei* EEs (*c*) and EErads (arrowhead) 6 months postimmunization (*d*).

as described (17, 18). The mean number of EErads per cm^2 visualized by DNA *in situ* hybridization was used to measure the efficiency of primaquine in eliminating persisting parasites in test liver samples. The percent elimination of EErads was compared with the controls in group I. At 7, 30, and 90 days posttreatment, rats were challenged with 1×10^4 or 1×10^5 spz. Loss of protective immunity was assessed by the presence of blood-stage infection up to 26 days after challenge. The presence or absence of parasitemia was determined from Giemsa-stained blood smears starting on day 4 and continuing through day 26 postchallenge. The mean number of irradiated parasites per cm^2 (EErads per cm^2) was calculated beginning 24 h after inoculation with γ-spz and continuing for 6 months. Each time point represents the mean number of EErads in 200 sections screened in each of three rats. DNA *in situ* hybridizations with a digoxigenin-labeled *P. berghei*-specific probe (15) were carried out for detection and quantitation of EEs in the liver sections on 5-μm frozen sections (16).

Immunization with EErads. Rats were immunized with 3×10^6 spz or γ-spz. Controls included sham-operated rats inoculated with mosquito salivary gland lysate by the intrahepatic method. After 24 h, 96 h, or 10 days, livers were removed, homogenized in an Omni homogenizer for 5 min at 20,000 rpm, and then freeze-thawed six times. Rats were then immunized with an aliquot of liver homogenate containing approximately 200,000 disrupted parasites as described (19). No adjuvant was used. Giemsa-stained blood smears were screened for parasitemia. No blood infection resulted in rats immunized with a homogenate that contained 24 or 46 h nonirradiated liver stages of the parasite. Rats were given four booster injections at 2-week intervals with the same homogenates. Immunized rats were challenged 7 days after the last booster injection with 1×10^3 or 1×10^4 spz injected intravenously and monitored for parasitemia for up to 26 days after being challenged. The reactivity of sera from immunized animals (groups I, II, and IV) were tested in an immunofluorescence assay against spz, EErads, 24-h EEs, and blood stages of the parasite.

RESULTS AND DISCUSSION

Rodents immunized with γ-spz of *P. berghei* (10 krad from a ^{137}C source) are protected from subsequent challenge with spz (20). We performed DNA *in situ* hybridization on liver sections with a nonradioactive *P. berghei*-specific DNA probe (15) to identify any EErads persisting in hepatocytes long after primary immunization. The number of EEs per cm^2 was quantitated from 600 liver sections made from Brown Norway rats at various intervals after immunization with either spz or γ-spz. The mean number of EErads per cm^2 observed at 48 h postimmunization was 128 ± 17. Subsequently, the number of EErads appeared to decrease by 26% (94 ± 19) by day 12 and by over 99% (0.5 ± 0.7) by day 30 postimmunization. Despite a substantial reduction in the number of EErads over time, some parasites were detected for up to 6 months postinoculation (Fig. 1). Similar trends of persistence were found in several strains of mice immunized with *P. berghei* γ-spz that had been attenuated to the same degree (10 krad; data not shown). Thus, the more sensitive approach of DNA *in situ* hybridization enabled the visualization of these small, persisting EErads *in vivo* which have been missed in previous immunofluorescent studies (21).

To determine the significance of persisting EErads in maintaining stage-specific protective immunity, the antimalarial drug primaquine, which has been shown to eliminate not only rapidly dividing liver-stage parasites (18) but also resident dormant forms of *Plasmodium vivax* from hepatocytes (17), was used to remove persisting parasites. Compared with untreated controls, greater than 99% of the EErads were eliminated when primaquine was applied 7 days after γ-spz inoculation (Table 1). Following primaquine treatment, the immunized hosts were challenged with spz on 7, 30, or 90 days posttreatment. Primaquine-treated rats were initially protected when challenged on day 7 (100% protected), but gradual loss of protection was observed in rats challenged 30 (58% protected) or 90 days (16% protected) after primaquine treatment. Thus, the presence of EErads for 7 days after

Table 1. Relationship between persisting EErads and protective immunity

Group	Treatment	EErads per cm^2, mean ± SD	spz challenge dose, $\times 10^{-3}$	Days posttreatment (no. protected/no. challenged)		
				7	30	90
I	PBS	118.31 ± 20	10	6/6	6/6	6/6
	PBS		100	6/6	6/6	6/6
II*	Primaquine	0.02 ± 0.1	10	6/6	4/6	1/6
	Primaquine		100	6/6	3/6	1/6
III	Primaquine†	0.00	10	0/3	0/3	0/3
	Primaquine		100	0/3	0/3	0/3

*A significant decrease was observed over time in group II. ($P < 0.001$ using exact methods of categorical data analysis. The EPIXACT/EGRET epidemiological data analysis package was used.)
†Nonimmunized controls.

immunization with γ-spz protected rats from subsequent spz challenge for at least 7 days after primaquine treatment. Rats treated with primaquine 1 month after immunization with γ-spz remained protected against spz challenge for up to 90 days (data not shown).

To further investigate the immunogenicity of EErads, rats were vaccinated with γ-spz, and the livers from these rats, which contained the EErads, were removed after 24 or 96 h or 10 days (Table 2). These livers were subsequently homogenized and inoculated i.p. into groups of 15 or 16 rats for each time point. Liver homogenates from rats infected with *P. berghei* spz at 24 and 46 h, as well as liver from uninfected animals, were used as controls. Neither inoculation with liver homogenates from these infected animals nor those from γ-spz produced a blood infection in the recipient animals. However, transfer of nonhomogenized, viable, infected hepatocytes did result in blood infection. More than 70% of rats immunized with a homogenate of either EErads (1, 4, or 10 days) or EEs (1 day) were protected when challenged 7 days following the last booster injection. In contrast, 75% of the rats immunized with a homogenate of mature EEs (48–72 h) developed parasitemia after spz challenge (Table 2). In a separate experiment, rats immunized with liver homogenates from the same time points as those in groups I–VI (Table 2) and challenged 30 days after immunization were not protected (data not shown). This short-lived protective immunity against spz challenge in these rats suggests that the presence of EErads and their antigens must be continually expressed to maintain long-lasting protective immunity. Serological analysis to determine the antigenic profile of EErads was performed by using antisera obtained from the immunized rats shown in Table 2. Sera from rats in groups I, II, and IV reacted with spz, early stages of EEs, and EErads. Sera from animals in group III reacted only with early stages of EEs and EErads. Overall, the data not only show that persisting EErads are immunogenic, but also confirm that irradiated and early liver stages of nonirradiated parasites—i.e., 24-h EEs—share common protective antigens.

The EEs are particularly attractive targets for malaria vaccine development because in addition to their stage-specific antigens they also share protein antigens with both spz and asexual blood stages. Furthermore, in contrast to spz, which remain in the circulation for a short period of time, complete development of EEs requires ≈48 h for *P. berghei* (5–7 days for *Plasmodium falciparum*). Therefore, immunity to EEs of the parasite abrogates blood infection, disease, and transmission of parasite to mosquitoes.

Table 2. Immunization of naive rats with EErads and protection against *P. berghei* spz challenge*

Group	Immunogen	spz challenge dose, $\times 10^{-3}$	No. protected/no. challenged	% protected
I	24-h EErads	1	7/8	88
		10	5/8	62
II	96-h EErads	1	7/8	88
		10	6/8	75
III	10-day EErads	1	7/8	88
		10	5/7	71
IV	24-h EEs	1	7/8	88
		10	5/8	62
V	46-h EEs	1	2/8	25†
		10	2/8	25
VI	Sham	1	0/9	0

*Sera from animals in groups I, II, and IV reacted with sporozoites, EErads, and 24-h EEs. Sera from animals in group III reacted with only EErads and EEs but did not react with fixed or unfixed *P. berghei* spz or blood stages.

†Group V has a significantly lower protection rate than that of groups I–IV ($P = 0.013$).

The hypothesis we propose is that γ-spz do not undergo complete schizogony to produce infectious merozoites but remain in the hepatocytes and express one or more stage-specific antigens. These antigens serve as immunogens that are essential for the development of immunity induced by γ-spz. We have shown that *P. berghei* DNA is present in the hepatocytes of animals that have received γ-spz 6 months previously. In addition, we have demonstrated that immunization of rats with 24- and 96-h-old EErads protects recipient animals from challenge with infectious spz. On the basis of these observations, we speculate that immunity to challenge infection seen in γ-spz-immunized animals is due to the continued presence of developmentally arrested parasites or their by-products.

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1. Mulligan, H. W., Russell, P. & Mohan, B. N. (1941) *J. Malar. Inst. India* **4,** 25–34.
2. Cochrane, A. H., Nussensweig, R. S. & Nardin, E. H. (1980) in *Malaria*, ed. Kreier, J. P. (Academic, New York), Vol. 3, pp. 163–202.
3. Spitalny, G. L. & Nussensweig, R. (1972) *Proc. Helminthol. Soc. Wash.* **39,** 508–514.
4. Egan, J. E., Weber, J. L., Ballou, W. R., Hollingdale, M. R., Majarian, W. R., Gordon, D. M., Maloy, W. L., Hoffman, S. L., Wirtz, R. A., Schneider, I., Woollett, G. R., Young, J. F. & Hockmeyer, W. T. (1987) *Science* **236,** 453–456.
5. Fries, L. F., Gordon, D. M., Schneider, I., Beier, J. C., Long, G. W., Gross, M., Que, J. U., Cryz, S. J. & Sadoff, J. C. (1992) *Infect. Immun.* **60,** 1834–1839.
6. Edelman, R., Hoffman, S. L., Davis, J. R., Beier, M., Sztein, M. B., Losonsky, G., Herrington, D. A., Eddy, H. A., Hollingdale, M. R., Gordon, D. M. & Clyde, D. F. (1993) *J. Infect. Dis.* **168,** 1066–1070.
7. Vanderberg, J. P., Nussensweig, R. S. & Most, H. (1968) *J. Parasitol.* **54,** 1175–1180.
8. Suhrbier, A., Winger, L. A., Costellano, E. & Sinden, R. E. (1990) *Infect. Immun.* **58,** 2834–2839.
9. Nussensweig, R., Vanderberg, J. P. & Most, H. (1969) *Mil. Med.* **134,** 1176–1182.
10. Hoffman, S. L., Franke, E. D., Rogers, W. O. & Mellouk, S. (1994) in *Molecular Immunological Considerations in Malaria Vaccine Development*, eds. Good, M. F. & Saul, A. J. (CRC, Boca Raton, FL), pp. 149–167.
11. Committee on Care and Use of Laboratory Animals (1985) *Guide for the Care and Use of Laboratory Animals* (Natl. Inst. Health, Bethesda, MD), DHHS Publ. No. (NIH) 85–23.
12. Pacheco, N. D., Strome, C. P. A., Mitchell, F., Bawden, M. P. & Beaudoin, R. L. (1979) *J. Parasitol.* **65,** 414–417.
13. Meis, J. F. G. M., Verhave, J. P., Jap, P. H. K. & Meuwissen, J. H. E. T. (1985) *Cell Tissue Res.* **241,** 353–360.
14. Scheller, L. F., Wirtz, R. A. & Azad, A. F. (1994) *Infect. Immun.* **62,** 4844–4847.
15. Ponzi, M., Janse, C. J., Pace, T., Dore, E., Reterink, T., Van den Berg, F. M. & Mons, B. (1990) *Mol. Biochem. Parasitol.* **41,** 73–82.
16. Van den Berg, F. M., Van Amstel, P. J., Janse, C. J., Meis, J. F. G. M. & Mons, B. (1991) *Histochem. J.* **23,** 109–115.
17. Peters, W., Robinson, B. J. & Milhous, W. K. (1993) *Ann. Trop. Med. Parasitol.* **87,** 547–552.
18. Boulard, Y., Landau, I., Miltgen, F., Ellis, D. S. & Peters, W. (1983) *Ann. Trop. Med. Parasitol.* **77,** 55–68.
19. Scheller, L. F., Stump, K. C. & Azad, A. F. (1995) *J. Parasitol.* **81,** 58–62.
20. Orjih, A. U., Cochrane, A. H. & Nussensweig, R. S. (1972) *Trans. R. Soc. Trop. Med. Hyg.* **76,** 57–61.
21. Sigler, C. I., Leland, P. & Hollingdale, M. R. (1984) *Am. J. Trop. Med. Hyg.* **33,** 544–547.