

IMMUNIZATION AGAINST RODENT MALARIA WITH CRYOPRESERVED IRRADIATED SPOROZOITES OF *PLASMODIUM BERGHEI*

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Abstract. The preparation and storage of *Plasmodium berghei* sporozoites for immunization purposes is described. The sporozoites were harvested from the salivary glands of infected mosquitoes, and maintained in cold tissue culture medium M199 with or without mouse serum. They were irradiated and frozen either at -75°C or in liquid nitrogen. After various periods sporozoites were thawed and injected into A/J mice. At the end of the immunization period the animals were challenged with infective sporozoites of *P. berghei* and monitored for parasitemia. It was found that the storage did not appreciably alter the ability of the irradiated sporozoites to induce protective immunity in the recipient animals. The highest protection (80-100%) was induced with sporozoites maintained in 10% serum and stored at -75°C .

It has been demonstrated in different experimental models that vertebrate hosts of malaria parasites can be protected against natural infections by prior immunization with sporozoite antigens (reviewed by Nussenzweig).¹ However, there are several practical difficulties still to be overcome before a mass immunization program with a sporozoite vaccine can be attempted. All successful immunizations to date have been achieved only with viable (though not necessarily infectious) sporozoites. The apparent requirement for viable parasites to induce protective immunity has caused some doubts whether sporozoite vaccine can be stored.^{2,3} A few attempts to preserve sporozoites in their infectious state have yielded conflicting results.^{4,5}

We report now on our success in immunizing mice with thawed irradiated sporozoites of *Plasmodium berghei* preserved frozen for up to 6 months at low temperatures, prior to their use.

MATERIALS AND METHODS

Animals

Adult female A/J mice (Jackson Memorial Laboratories, Bar Harbor, Maine) were used in all the experiments.

Sporozoites

Plasmodium berghei, NK 65 strain, was used throughout the studies. The sporozoites of this ro-

dent malarial parasite were obtained from the salivary glands of female *Anopheles stephensi* mosquitoes which had fed on *P. berghei*-infected hamsters 18 days prior to their dissection. Details concerning the laboratory maintenance of this strain of rodent malarial parasite, including its cyclical transmission and the characteristics of the infection in A/J mice have been described by Vanderberg et al.⁶

Maintenance medium for sporozoites

Throughout the experiments the sporozoites were suspended in tissue culture medium M199 (Grand Island Biological Company, Grand Island, New York), with or without serum supplement. The serum-supplemented medium was prepared by adding one part of normal A/J mouse serum to nine parts of M199 to obtain a 10% serum dilution. Mouse serum was used in this medium in order to avoid sensitization of the experimental animals during the course of sporozoite immunization.

Harvesting, irradiation, freezing and storage of sporozoites

The salivary glands of the infected mosquitoes were dissected out and collected in the appropriate maintenance medium. To free the sporozoites, which were maintained at 4°C throughout the initial preparation period, the glands were gently triturated in a loose-fitting all glass tissue grinder. The sporozoites were then separated from the

TABLE 1

Experimental design for the immunization of mice against *Plasmodium berghei* infection with cryopreserved irradiated sporozoites

Experiment	Number of sporozoites			Number of inoculations	Days stored frozen prior to inoculation	
	Per inoculation		Total		First dose	Last dose
	Primary	Boosters				
I	7×10^4	1×10^4	1.1×10^5	5	5	33
II	7×10^4	1×10^4	1.1×10^5	5	7	35
III	7×10^4	1×10^4	1.1×10^5	5	31	60
IV	7×10^4	1×10^4	1.1×10^5	5	42	68

mosquito tissue debris by centrifuging the triturated glands at 500 rpm for 3 min and collecting the parasite-containing supernatant. For a maximum yield, the extraction process was repeated twice. The concentration of the sporozoites in the pooled supernatants was determined by counting the parasites in a hemocytometer.

The parasite suspension was then adjusted to concentrations varying from 1.5×10^5 to 1.05×10^6 sporozoites/ml of medium, depending on the desired immunizing dosage. Aliquots of 0.4 ml of the sporozoite suspension were distributed in 2-ml freezing vials (Cooke Laboratory Products, Virginia). After being irradiated at 15 K rads in a gammator (Radiation International, Inc.) the vials were transferred into a freezer kept at -75°C or into a liquid nitrogen tank for storage.

Immunization procedure

The immunization schedule followed in this study was essentially that established by Nussen-zweig et al.⁷ for the immunization of rodents with irradiated sporozoites of *P. berghei*. Briefly, the procedure involves five inoculations of the sporozoite vaccine administered intravenously at 7- to 14-day intervals. Immediately before the injection into the experimental animals the frozen vial of sporozoites was removed from storage and quickly thawed by hand. A vigorous rolling of the tube between the palms usually resulted in a complete thaw of the suspension within 2 min. The suspension was then diluted with cold medium with or without serum, so that the appropriate inoculum for each mouse was contained in 0.2 ml of the medium. All suspensions were thoroughly mixed and held at 4°C during inoculation. Delay between the thawing and the injecting of the vaccine was avoided and no thawed preparation was ever refrozen.

Challenge infection

Between 5 and 14 days after receiving the final immunizing dose, the animals were challenged intravenously with 1×10^4 infective sporozoites of *P. berghei*. Non-immunized mice serving as controls were similarly inoculated. Starting from day 4 after the challenge, the animals were examined on alternate days for detectable parasitemia in Giemsa-stained blood smears. All patent infections resulting from challenge usually appeared within 10 days in both the immunized and non-immunized control mice. Therefore, animals which did not show a detectable parasitemia within 14 days following the challenge were considered to be completely protected.

RESULTS

The experimental design followed for the immunization with cryopreserved γ -irradiated sporozoites of *P. berghei* is depicted in Table 1. In each experiment we used the same batch of sporozoites for the whole immunization procedure, i.e., one preparation of irradiated sporozoites after being distributed in vials and maintained at low temperatures, was used after different time periods for the primary as well as the booster immunizations. The vaccines in Experiments I and II were stored for at least 5 days prior to their use. In Experiments III and IV the minimum storage period was extended to 31 days. The minimum storage period in the context of these experiments is the time in days the first immunizing dose of sporozoites was kept frozen before injection into the recipient animals. The maximum storage period, i.e., the length of time that the last immunizing dose was kept frozen, was 33 and 35 days for Experiments I and II, and 60 and 68 days for Experiments III and IV, respectively.

TABLE 2

Number and percentage of mice protected against challenge after immunization with cryopreserved irradiated sporozoites of *Plasmodium berghei*: Effect of serum and temperature

Experiment	Vaccine preserved at -75°C		Vaccine preserved in liquid nitrogen	
	With serum	Without serum	With serum	Control
I	5/5 (100)*	2/5 (40)	3/5 (60)	0/5 (0)
II	5/5 (100)	5/5 (100)	5/5 (100)	0/5 (0)
III	11/13 (85)	8/13 (62)	7/13 (54)	0/13 (0)
IV	13/13 (100)	12/13 (92)	6/13 (46)	0/11 (0)
Total	34/36 (94)	27/36 (75)	21/36 (58)	0/34 (0)

* Number protected/number challenged (percent protected).

In all the experiments shown in Tables 1 and 2 the primary immunizing dose consisted of 7×10^4 irradiated sporozoites, while the boosters contained 1×10^4 sporozoites. Thus, each experimental mouse received a total of 1.1×10^5 immunizing sporozoites in five inoculations.

Table 2 illustrates the results obtained when mice immunized with irradiated sporozoites cryopreserved in different conditions were challenged with 1×10^4 infective sporozoites of *P. berghei*. In most of the experiments immunization with irradiated sporozoites frozen and stored at -75°C in the presence of 10% normal mouse serum consistently resulted in protection of all the animals against sporozoite-induced infection. In the four experiments consisting of 36 mice immunized with irradiated sporozoites preserved in serum at -75°C , 34 (i.e., 94%) of the animals were protected against challenge infection. Immunization with irradiated sporozoites cryopreserved at -75°C without serum produced variable levels of protection which ranged from 40–100% in the different experiments, with a mean of 75% of the animals being protected. A similar variation in the level of protection against challenge was observed when animals were immunized with irradiated sporozoites maintained in 10% normal mouse serum but stored in liquid nitrogen (-196°C). In repeated experiments, 21 of a total of 36 mice were protected by immunization with liquid nitrogen-preserved irradiated sporozoites maintained in serum.

It should be noticed that except for the differences in the length of time the sporozoites were stored frozen, the four experiments summarized in Tables 1 and 2 are similar. The protection we observed is attributed to the immunizations because the strain of mice we used is fully susceptible to sporozoite-induced *P. berghei* malarial infection. All the non-immunized control animals

developed malaria from challenge with the same preparation of infective sporozoites used for the experimental animals.

DISCUSSION

We have developed a simple method for the preservation of irradiated sporozoites of *P. berghei* for immunization of rodents against malaria infection. The method involves freezing and storage of the sporozoites at low temperature. The procedure does not require any cryoprotectant that might be toxic for the animals being immunized. However, addition of 10% serum to the maintenance medium is necessary for a better preservation of the vaccine, since this consistently produces a high percentage of protection.

Serum has been known to have some cryoprotective effect of frozen cells.⁸ Jeffery and Rendtorff⁴ have stored sporozoites of various human malaria parasites in serum at low temperature without much loss of the parasites' infectivity to experimental monkeys. It must, however, be stressed that there is as yet no known correlation between the infectivity and the immunogenicity of plasmodial sporozoites.

We cannot say at this time whether the irradiation of the sporozoites before freezing is of any significance to their successful cryopreservation, since we did not immunize with sporozoites irradiated following freezing and thawing. There is a need for irradiation because the freezing and subsequent thawing does not always completely abolish the sporozoite infectivity. Thus, when we tried to immunize animals with non-irradiated sporozoites, frozen in the presence of serum, some of the animals developed patent infections during the course of the immunization (data not shown).

In our present studies, the freezing and storage temperature for the sporozoites seemed to be an

important factor in preserving the immunogenicity. Our data suggest that it is better to freeze and store the *P. berghei* sporozoite vaccine at -75°C than in liquid nitrogen (-196°C).

The level of protection obtained upon immunization of animals with irradiated sporozoites stored at -75°C in the presence of serum does not differ from that obtained following the standard procedure for immunization with irradiated sporozoites, which involves the injection of the vaccine intravenously immediately after its preparation. This procedure requires about 1.5×10^5 irradiated sporozoites in M199 administered in five injections to establish a high degree of protection in mice. However, we have found that the injection of only two doses of 1×10^4 sporozoites of *P. berghei*, harvested and irradiated in the presence of 10% normal mouse serum, consistently induces complete protection in 80–100% of the immunized animals (Orjih, unpublished results). In a preliminary experiment in which we immunized a group of mice with two doses of 1×10^4 irradiated sporozoites maintained in serum and cryopreserved at -75°C , only 40% of the animals were protected against challenge infection. We have yet to determine whether this lower level of protection is due to the cryopreservation or the differences in the immunogenicity of sporozoite preparations.

In other experiments not documented in the Tables, we found that five injections of 1×10^4 irradiated sporozoites maintained in 10% serum and stored at -75°C , i.e., less than one half of the total sporozoites used in Experiments I to IV, yielded 90–100% protection against challenge. We also found that the storage of irradiated sporozoites in serum at -75°C for 6 months did not appreciably reduce the capability of the vaccine to induce protective immunity. Eighty percent of the animals immunized with the vaccine cryopreserved for 6 months were protected against challenge. These experiments still have to be extended in order to determine how long irradiated sporozoites maintained in serum can retain their ability to induce protective immunity when stored at -75°C . However, based on the data we have presented, the storage period certainly can be for several months.

There is an obvious need for an adequate technique for the preservation of sporozoites, since a sporozoite vaccine is being considered as a potential immunoprophylactic approach against malaria. Such a vaccine would unquestionably be in-

tended for use in malaria-endemic areas, and thus, must be preservable to permit its application in the field. Even at this initial stage of the development of this vaccine, there is a great need for a method for its storage, which will permit comparative studies on the immunogenicity of different sporozoite preparations. It is well known that different batches of *P. berghei* sporozoites differ in their ability to induce protective immunity in experimental animals. For example, in an early immunization study involving the administration of a single dose of irradiated sporozoites Nussenzweig et al.⁹ found that the percentage of protected animals varied from 27–86% in different experiments. The basis of this variation could thus far not be investigated, because each preparation of sporozoites had to be used shortly after dissection. Storage now permits the selection of highly immunogenic vaccine preparations for immunization purposes. This may reduce the need for the several immunizing doses of irradiated sporozoites presently necessary for the establishment of protective immunity.

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REFERENCES

1. Nussenzweig, R. S., 1977. Immunoprophylaxis of malaria: Sporozoite-induced immunity. *Adv. Exp. Med. Biol.*, 93: 75–97.
2. Cohen, S., 1978. Development of a malaria vaccine. *J. R. Soc. Med.*, 71: 476–478.
3. Miller, L. H., 1977. A critique of merozoite and sporozoite vaccines in malaria. *Adv. Exp. Med. Biol.*, 93: 113–119.
4. Jeffery, G. M., and Rendtorff, R. C., 1955. Preservation of viable human malaria sporozoites by low temperature freezing. *Exp. Parasitol.*, 4: 445–454.
5. Befort, J., 1968. The effects of low temperature preservation on the viability of the sporozoites of

- Plasmodium berghei*. *Ann. Trop. Med. Parasitol.*, 33: 495-498.
6. Vanderberg, J. P., Nussenzweig, R. S., and Most, H., 1968. Further studies on the *Plasmodium berghei*-*Anopheles stephensi*-rodent system of mammalian malaria. *J. Parasitol.*, 54: 1009-1016.
 7. Nussenzweig, R. S., Vanderberg, J. P., and Most, H., 1969. Protective immunity produced by the injection of X-irradiated sporozoites of *Plasmodium berghei*. IV. Dose response, specificity and humoral immunity. *Milit. Med.*, 134 (suppl.): 1176-1182.
 8. Mazur, P., 1970. Cryobiology: The freezing of biological systems. *Science*, 168: 939-949.
 9. Nussenzweig, R. S., Vanderberg, J. P., Most, H., and Orton, C., 1967. Protective immunity produced by the injection of X-irradiated sporozoites of *Plasmodium berghei*. *Nature*, 216: 160-162.