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Low-temperature preservation of sporozoites of *Plasmodium berghei**

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Large numbers of biologically active sporozoites are needed as a source of potential antigen in the development of a malaria vaccine and the most practical method of accumulating sufficient numbers of these forms would be to freeze and store them at low temperature. The purpose of this work was to determine the feasibility of preserving the infectivity of frozen and thawed sporozoites. The results indicate that sporozoites of Plasmodium berghei *exhibit a typical response to freezing over a wide range of cooling rates; the distribution around the optimum was a normal one and both the magnitude and position of the peak of their infectivity depended upon the preservative used. The optimum cooling rate with preservatives of high relative molecular mass was between 20 °C and 60 °C per min, but varied with the preservative used. A new apparatus was designed and built to yield reproducible controlled cooling rates over the range studied. A comparison of various commonly used preservatives showed mouse serum alone to be effective, but the combination of serum and hydroxyethyl starch proved to be the best cryopreservative of those examined.*

The successful freezing of *Plasmodium* sporozoites was first reported by Jeffery & Rendtorff (*1*) who found that sporozoites from several strains of human malaria, frozen in plasma, retained their infectivity while those frozen in saline solution did not. Bafort (*2*) referred to this study as the only successful attempt to freeze sporozoites to date. He concluded from his own work on freezing sporozoites of rodent malaria that they were quite difficult to freeze, because only 2 out of 26 samples retained infectivity. Since we plan to use frozen sporozoites of *P. berghei* as a source of antigen in the development phase of a malaria vaccine, it is important that these forms remain biologically intact. For this reason, infectivity is used as the criterion of physiological integrity. A systematic study, treating the cooling rate, warming rate, and type and concentration of preservative as independent variables, is required to establish the feasibility and optimum conditions for storing sporozoites at low temperature (*3, 4*).

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METHODS

Determination of the optimum cooling rate requirements of Plasmodium berghei *sporozoites*

Sporozoites were isolated with mouse serum as described by Pacheco et al. (*5*) or in the absence of mouse serum. They were resuspended for freezing in 1.0-ml volumes (15 x 100 mm tubes) in 100% mouse serum, or in a mixture containing 75 ml of dimethyl sulfoxide (DMSO) and 925 ml of serum per litre, or in Medium 199 (M-199) alone. All cell suspensions were adjusted to a concentration of 4×10^5 sporozoites per ml, as determined by duplicate haemacytometer counts. Unfrozen, control suspensions of 4×10^4 sporozoites were injected in 0.1-ml volumes via the tail vein into male NIH/NMRI mice, which were approximately 6 weeks old; all preparations were kept at 0 °C prior to injection. The percentage infectivity was determined from blood films stained with Giemsa stain on days 7, 9, and 16 after inoculation.

The samples to be frozen were first brought to approximately −3 °C in an insulated bath containing absolute ethanol and equipped with a coil through which liquid nitrogen was circulated. The bath was further equipped with a stirrer and each sample was supplied with a copper–constantin thermocouple (22-gauge) connected to a Honeywell Electronik 112 multipoint recorder. In one sample tube, an additional thermocouple leading to a Honeywell Visicorder 906C was joined to the thermocouple attached to the multipoint recorder to obtain a continuous cooling curve record during freezing. When the samples

reached –3 °C, crystallization was induced by seeding with a column of frozen medium contained in a Pasteur pipette to avoid different degrees of supercooling.

After the phase change had been completed (approximately 5 min) and the samples had returned to –3 °C, they were ready to continue being cooled in one of three ways: (*a*) Cooling rates from 0.2 °C to 30 °C per min were achieved using apparatus as described by Leibo et al. (*6*). (*b*) Rates from 30 °C to 100 °C per min were obtained as described by Leef et al. (*7, 8*). The apparatus used was designed and built for this study and consists of a cold alcohol reservoir connected to a delivery tube where the flow rate is controlled by a ball valve equipped with stops. Cold alcohol from the reservoir displaces the warmer alcohol in the sample container and the rate of this displacement determines the cooling rate. (*c*) A cooling rate of 400 °C per min was obtained by immersing the sample directly into liquid nitrogen (–196 °C). All cooled samples were thawed by immersion and rapid agitation in a 37 °C water bath (approximately 300 °C per min) and inoculated into mice at a dose of 4 x 10^4 sporozoites to assess their infectivity as described above.

Survey of preservatives

The preservatives compared fall into two broad classes—those with low and those with high relative molecular mass (RMM). The compounds with low relative molecular mass were DMSO, RMM 78.1, adjusted to concentrations of 50, 75, 100, or 150 ml/litre, and glycerol, RMM 92.1, adjusted to concentrations of 50, 100, or 150 ml/litre. The preservatives with high relative molecular mass were: whole mouse serum, adjusted to the concentrations of 92.5 or 100%; polyvinyl pyrrolidone (PVP), RMM 40 000, adjusted to concentrations of 50, 100, or 150 g/litre, and hydroxyethyl starch (HES), RMM 150 000, adjusted to concentrations of 50, 100, or 150 g/litre. The HES was supplied by Dr F. J. Lionetti, Center for Blood Research, Boston, MA, USA. All preservative solutions with the exception of DMSO were made at twice the concentrations needed in Medium-199 (M-199) and equal volumes of those solutions were added to equal volumes of sporozoites suspended in whole serum. The DMSO solutions were prepared by mixing appropriate volumes of DMSO and mouse serum, so that in the case of a 100-ml/litre DMSO solution, the concentration of the serum was 90%. All sporozoites were isolated, frozen, thawed, and the infectivity determinations made as described in the section above dealing with requirements for optimal cooling rates.

RESULTS

Fig. 1 shows a comparison between the infectivity of sporozoites isolated and maintained in the presence or absence of mouse serum before injection into mice. The time before inoculation was the same for both groups, approximately 3 hours, yet the infectivity of the sporozoites in the absence of serum was only 10% as compared with nearly 100% infectivity of the samples suspended in serum.

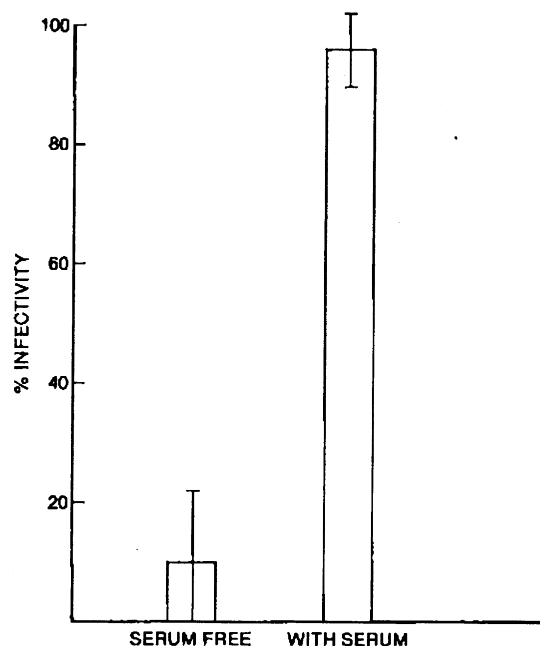

Fig. 1. The effect of the presence or absence of whole mouse serum on the infectivity of *P. berghei* sporozoites. The vertical lines represent the standard deviations.

Fig. 2 shows the effect on the infectivity of sporozoites of holding in 100% mouse serum at 0 °C for various times before inoculation. Up to 3.5 hours, there was no discernible effect, but sample infectivity declined to approximately 11% by 24 hours.

The results of the initial optimal cooling rate determinations are summarized in Fig. 3. Each point represents a mean of at least three experiments where each experimental group contained 10 mice. Fig. 3 indicates that a 75-ml/litre DMSO solution is most effective at a cooling rate of 1 °C per min, but that it is not nearly as effective a cryopreservative as is whole mouse serum. DMSO concentrations of 50, 100 and 150 ml/litre were also examined but they were even

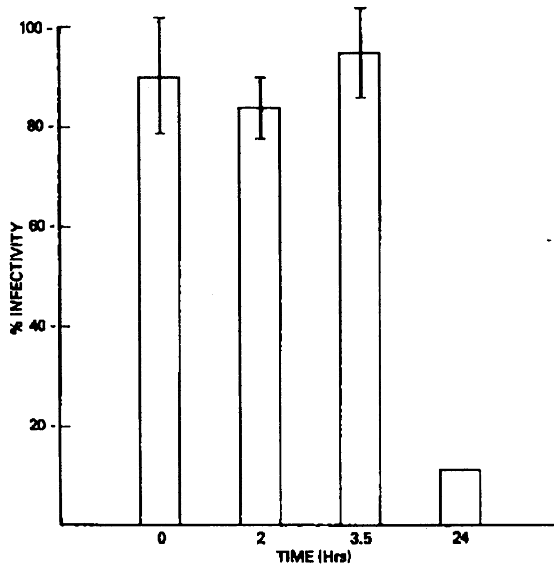

Fig. 2. The effect of incubation at 0°C on the infectivity of sporozoites of *P. berghei*. The vertical lines represent the standard deviations.

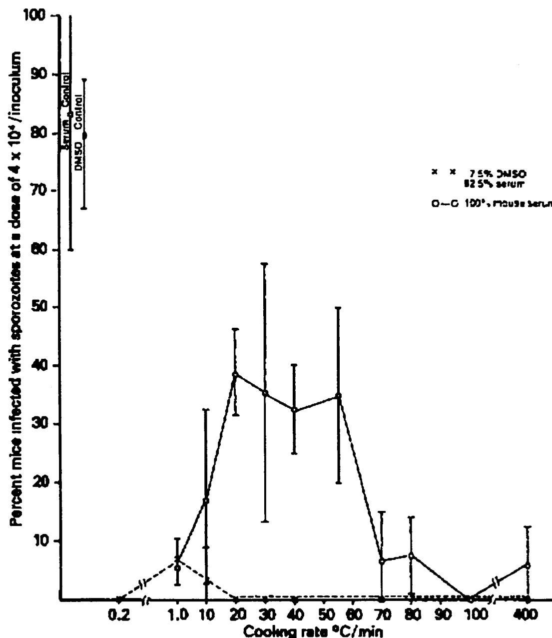

Fig. 3. A comparison between DMSO and mouse serum in the low-temperature preservation of *P. berghei* sporozoites. The two single points (controls: top left) are the infectivity of unfrozen sporozoites incubated in either whole mouse serum or 100 ml/litre DMSO in mouse serum. All other points relate to frozen sporozoites. The vertical lines represent the standard deviations.

less effective than the 75-ml/litre solution. Whole mouse serum is most effective over the cooling rates of 20°C to 60°C per min. Furthermore, maximum infectivity of 40% was retained following freezing with serum, whereas it was 10% with DMSO. Fig. 3 also shows that DMSO had no deleterious effects on sporozoites when they were incubated in its presence, but not frozen.

The results of a comparison between various preservatives tested are summarized in Fig. 4. They suggest that HES is the best preservative thus far examined. The percentage infectivity of sporozoites following freezing for the preservatives tested was as follows: HES, 65%; PVP, 55%; serum, 40%; and DMSO, 10%. The data for glycerol are too preliminary for comparison, but this material appears to be better than DMSO. Although other concentrations of the various preservatives were examined, only the results for the concentration yielding the highest infectivity are reported.

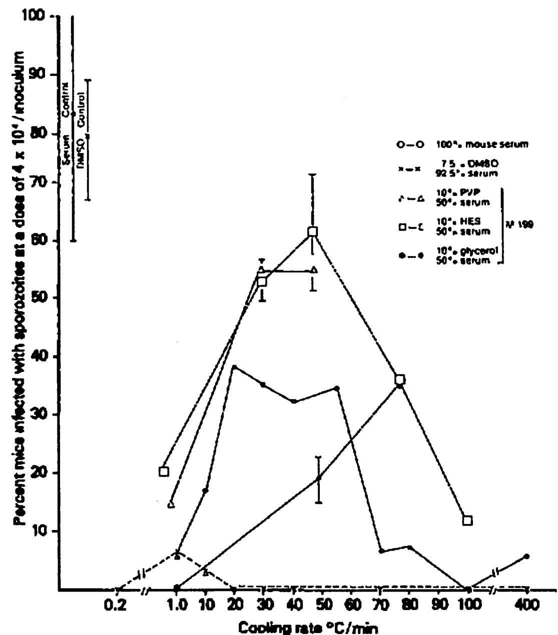

Fig. 4. A preliminary comparison between preservatives with low and high relative molecular mass in the low-temperature preservation of *P. berghei* sporozoites. The vertical lines represent the standard deviations.

DISCUSSION

It has been established that different types of cell require different cooling and warming rates to retain optimum viability after freezing (*9, 10, 11*). Mazur

explained the parameters involved in the optimum cooling rate (*9*, *10*) and his work has been substantiated by others (*12*).

In this study, the effects of various cooling rates and different preservatives on sporozoites during freezing had to be determined in the presence of serum because in its absence sporozoites lose their infectivity (Fig. 1); therefore, it became a necessary constituent of all freezing media. In the presence of serum, the sporozoites were quite stable at 0 °C for at least 3.5 hours. It is unlikely that the processing time for samples to be frozen would ever extend beyond that time, so that, although there is a significant decrease in the infectivity following incubation for 24 hours at 0 °C, incubation times beyond 3.5 hours were not examined.

We have shown that the optimum cooling rate for sporozoites protected with preservatives of high relative molecular mass lies in the range of 20 °C to 60 °C per min. The highest degree of protection was given by a combination of HES and whole mouse serum. It is apparent from Fig. 3 and 4 that the optimum cooling rate can vary greatly with the preservative used. For this reason, the effects of various cooling rates must be examined with any preservative studied. If only a single rate is examined, the rate may be far from optimum, even though it was the best rate to use with another preservative.

The evidence presented above establishes the feasibility of storing sporozoites at low temperature. This approach, if successful, would mean that such material could be accumulated in quantity and kept for long periods. Recently, Strome et al. (*13*) showed that malaria parasites of the erythrocytic cycle brought to –196 °C could remain at that temperature for at least 10 years without loss of biological activity, and although data on long-term storage of sporozoites are not available, there is no reason to suppose that this stage will behave differently when stored in liquid nitrogen.

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RÉSUMÉ

CONSERVATION A BASSE TEMPÉRATURE DES SPOROZOÏTES DE *PLASMODIUM BERGHEI*

Les travaux de mise au point d'un vaccin antipaludique qui font appel aux sporozoïtes comme source possible d'antigène nécessitent de grandes quantités de parasites biologiquement actifs. La méthode la plus pratique pour disposer de ces stades du parasite en suffisance serait de les congeler et de les stocker à basse température. Mais il faut que soit d'abord confirmée la possibilité de préserver le pouvoir infectant chez les sporozoïtes congelés puis décongelés, et c'est à quoi tend la présente étude. Elle a permis de constater chez les sporozoïtes de *Plasmodium berghei* une réponse à la congélation qui varie selon la vitesse de refroidissement dans une gamme de valeurs étendue; il s'établit une distribution normale de part et d'autre d'une vitesse optimale, et le taux maximum de pouvoir infectant et son maintien pour une gamme plus ou moins large de vitesses de refroidissement dépendent de l'agent conservateur utilisé. Avec des agents conservateurs de masse moléculaire relative élevée, la vitesse de refroidissement optimale se situe entre 20°C et 60°C par minute, mais varie fortement selon l'agent employé. Dans un nouvel appareil spécialement conçu à cet effet, le refroidissement peut s'effectuer à des vitesses contrôlées et reproductibles entre les deux limites ainsi fixées. Une comparaison entre les divers agents conservateurs d'usage courant a montré que le sérum de souris était le seul qui soit efficace et, d'après les expériences faites, la combinaison de ce sérum avec de l'amidon hydroxyéthylé constitue le meilleur agent de cryoconservation.

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