Preservation of intraerythrocytic forms of malarial parasites by one-step and two-step cooling procedures

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Ring, trophozoite, and schizont stages of Plasmodium knowlesi were cooled in dimethyl sulfoxide either by direct immersion in liquid nitrogen or by a two-step method in which the cells were held at temperatures slightly below 0°C for different lengths of time before they were cooled to −196°C. After the direct plunge treatment, thawed trophozoites and schizonts were found to be extensively damaged. Their survival was markedly increased by holding them at −31°C for 30 min before plunging them into liquid nitrogen. Freeze-substitution showed that cells cooled by the two-step procedure were grossly shrunken and had relatively few intracellular ice cavities. Large amounts of ice formed in trophozoites and schizonts preserved by direct immersion in liquid nitrogen. The two-step protocols investigated did not improve the survival of ring-stage parasites, 25–50% of which survived rapid cooling to −196°C. Infected cell agglutination tests were carried out with frozen and thawed schizonts. Variant specificity was demonstrated with cells that had been plunged directly to −196°C, but cells cooled by the two-step method tended to agglutinate spontaneously.

For the optimal preservation of malarial parasites at low temperatures, the following factors should be determined: the best cryoprotectant and its concentration, the most favourable freezing conditions, the best rate of thawing, and a convenient method of returning the cells to physiological conditions. Various attempts to preserve malarial parasites at low temperatures have been reported since the 1930s but a methodology to take account of the factors mentioned above has not been established.

Knowledge of these factors is important for the cryopreservation of any cell system because higher yields of cells may be obtained by adapting the method to each cell type (I). Also, some procedures result in selective destruction when mixed cell populations are frozen (2), or can alter cell surface characteristics (3). These observations are relevant to one of the main objectives of the cryopreservation of malarial parasites, namely, the maintenance in stored form of a viable, homogeneous parasite population without alteration of its antigenic or other biological characteristics.

Preliminary investigations to establish the best freezing conditions for the cryopreservation of simian blood infected with Plasmodium knowlesi are described below. The two-step cooling procedure that we employed can separate the different sets of conditions that lead to cell injury (4). In this procedure, samples are cooled to a selected temperature (e.g., −25°C) and are held at that temperature for different lengths of time before being plunged into liquid nitrogen. At the holding temperature, cells may acquire “protection” against damage caused by rapid cooling in liquid nitrogen and subsequent thawing. Damage incurred at the holding temperature itself can be assessed by thawing a duplicate set of samples. The extent of protection against damage during cooling to −196°C and thawing can be assessed by comparing the damage in the plunged sample to that in the sample subjected to the holding temperature only.

In our experiments, damage incurred by frozen erythrocytes was determined from the percentage lysis on thawing and from cell agglutination. The viability of frozen and thawed intraerythrocytic parasites was evaluated by their subsequent growth and multiplication in tissue culture. Our findings indicate that the optimal freezing conditions for red
cells differ from those for the various developmental stages of the parasite.

**MATERIALS AND METHODS**

**Host–parasite system**

Rhesus monkeys were infected by blood passage of the Nuri strain of *P. knowlesi* and parasitaemia was monitored by means of Giemsa-stained blood smears. Blood obtained by venepuncture was diluted in heparinized modified Ringer’s solution (5) to give 17 IU of heparin per ml. Antigenic variants of *P. knowlesi* were isolated as described by Brown & Brown (6).

**Schizont-infected cell agglutination test**

Red cells infected with schizonts were concentrated by the low speed sucrose gradient centrifugation method of Williamson & Cover (7). Agglutination tests were carried out as described by Brown & Brown (6).

**Freezing and thawing procedure**

As whole blood preparations tended to clot after thawing, washed erythrocytes resuspended to 50% haematocrit in uninfected monkey serum (NMS) were usually prepared for freezing. Blood reconstituted in this way was diluted by the rapid addition of an equal addition of a 200-ml/litre ice-cold solution of dimethyl sulfoxide (DMSO) in Ringer’s solution. After mixing, 0.5-ml samples were dispensed into screw-capped Sterilin polypropylene tubes for freezing. Some paired samples were protected against injury on immersion in liquid nitrogen by prefreezing at higher temperatures (e.g., −25°C) in a refrigerated alcohol bath—the two-step technique. Subsequently, one tube from each pair was plunged into liquid nitrogen. To investigate damage incurred during the initial freezing, the other tubes were thawed and stored on ice. Thawing was carried out by rapidly transferring tubes to a water bath at 37°C and gently agitating them for 1 min. Tubes in liquid nitrogen were thawed in a similar manner after 90 min of freezing. Controls included unfrozen cells plus DMSO as well as cells plus DMSO plunged directly into liquid nitrogen. After thawing, the cells were centrifuged at 500 g for 2 min and then washed by resuspension and centrifugation sequentially in 1-ml volumes of 10% sorbitol, 5% sorbitol, and finally Ringer’s solution. Damage to the cells in the pellets was minimized by the slow addition of the washing fluids with gentle agitation. The optical density (at 412 nm) of the supernatant was measured after each centrifugation. The cell pellets were finally resuspended in NMS and the viability of the parasites was tested in a microtissue culture tray.

**Tissue culture**

Washed, parasitized red cells were resuspended in NMS and cultured in triplicate in microtissue culture trays as described by Phillips et al. (8), and 37 kBq of ³H-isoleucine (629 GBq/mmol) was added to duplicate wells for each sample. Cultures were usually harvested 24 h later and samples were taken for microscopic examination as well as for measurement of isotope incorporation. Fresh red cells were not added to cultures of frozen and thawed blood.

**Scintillation counting**

Cells were transferred from tissue culture trays to glass tubes (30 × 6 mm) and washed three times in Ringer’s solution. The cells, suspended in a small volume of fluid, were then placed in glass scintillation vials and two drops of 30% H₂O₂ were added. Two hours later, the samples were solubilized in 0.5 ml NCS (Amersham–Searle). The vials were incubated at 56°C for 1 h and 6 ml of toluene-based scintillation fluid were then added. Quenching was monitored by means of the external standard of a Packard 2420 scintillation counter.

**Freeze substitution and electron microscopy**

Following the addition of cryoprotectant, infected red cells were pelleted by centrifugation and most of the supernatant fluid was removed. The cell pellets were cooled to an appropriate temperature in unstoppered polypropylene Sterilin tubes before being plunged into liquid nitrogen. Freeze substitution at −80°C and electron microscopy were carried out as described by Walter et al. (9).

**RESULTS**

**Cryoprotectant**

Preliminary experiments with unfrozen schizonts and ring-stage parasites showed that the cryoprotectants, DMSO and glycerol, were toxic at a final concentration of 200 ml/litre. Consequently, 100-ml/litre DMSO was used as the cryoprotectant in all the experiments described below.

**Freezing of red cells containing trophozoites and schizonts**

Blood containing 20% infected erythrocytes (96% of the parasites were trophozoites and schizonts) was
mixed with an equal volume of 200-ml/litre DMSO. Duplicate samples were frozen at -25°C or -32°C and held at that temperature for 5, 10, 30, or 60 min before they were cooled to -196°C. Lysis data from thawed samples (Fig. 1A) showed that some damage occurred to red cells held at higher freezing temperatures before immersion into liquid nitrogen. This effect was more marked at -32°C than at -25°C and tended to increase with time at that temperature. There was very little lysis of cells that had been plunged directly into liquid nitrogen and subsequently thawed.

Freezing conditions that yielded parasites with the least amount of obvious morphological damage were the converse of those described above for red cells. The survival of undamaged parasites following immersion in liquid nitrogen increased with the length of time (up to 30 min) that they had previously been held at -25°C or -32°C (Fig. 1B). Direct immersion in liquid nitrogen damaged nearly all the parasites, whereas prior freezing at -32°C for 30 min gave the highest yield of virtually undamaged parasites with minimal morphological changes.

That the two-step method protected trophozoites and schizonts against damage caused by rapid freezing in liquid nitrogen was confirmed by subsequent culture of the cells (Fig. 2A). After schizogony in vitro, parasites that had been held at -25°C for 10 min prior to immersion in liquid nitrogen yielded a new brood of parasites equal in number to those produced by the unfrozen sample. Cells held at -32°C for 30-60 min gave the best multiplication after thawing from -196°C and restored the percentage of infected erythrocytes to that of the original inoculum.

The incorporation of ³H-isoleucine followed similar patterns (Fig. 2B). Uptake was always lower in thawed cells recovered after direct immersion in liquid nitrogen, but reached the same levels as in unfrozen controls when cells were protected by the two-step method. Although the parasite density in all groups was excessively high for this tissue culture method, and this probably accounted in part for the low level of multiplication obtained in the experiment, differences between the direct plunge and the two-step protective treatments could clearly be seen.

The results of a similar two-step experiment with blood containing 11.5% parasitized erythrocytes (93% of the parasites were trophozoites and schizonts) are shown in Fig. 3. This experiment confirmed that -24°C was too high a temperature for the first freezing step and that yields of viable cells were increased if they were held at about -31°C for 30-60 min before immersion in liquid nitrogen.

In both experiments described above, cultures of thawed blood that had been frozen at temperatures

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Fig. 1. Preservation of trophozoites and schizonts of *P. knowlesi* by direct cooling to -196°C or by rapid cooling from holding temperatures of -25°C and -32°C. (A) Percentage haemolysis. (B) Parasites with minimally altered morphology. P = plunged; NOP = not plunged.

Fig. 2. Tissue culture of trophozoites and schizonts of *P. knowlesi* preserved by direct cooling to -196°C or by two-step cooling. (A) The number of parasites produced after one cycle of multiplication in vitro. (B) The incorporation of ³H-isoleucine following parasite multiplication in vitro. P = plunged; NOP = not plunged.
as low as -30°C before being plunged into liquid nitrogen gave better multiplication of the parasites than parallel samples that were frozen to the holding temperature and then thawed directly without being plunged into liquid nitrogen. The reason for this difference has not been established.

Fig. 3. Tissue culture of trophozoites and schizonts of *P. knowlesi* preserved by direct cooling to -196°C or by rapid cooling from holding temperatures of -24°C, -28°C, and -31°C. (A) The number of parasites produced after one cycle of multiplication *in vitro.* (B) The incorporation of 3H-isoleucine following parasite multiplication *in vitro.* P = plunged; NOP = not plunged.

Fig. 4. Agglutination of schizont-infected red cells before freezing, after direct cooling to -196°C, and after rapid cooling from a holding temperature of -31°C. The sera titrated were as follows: (A) uninfected monkey serum, (B) antiserum from a monkey infected with the homologous antigenic variant, (C) antiserum from a monkey immunized with homologous variant plus adjuvant, and (D) antiserum from a monkey infected with a heterologous variant.
Antigenicity of thawed schizonts

Schizonts purified on sucrose gradients were washed before being mixed with an equal volume of 200-ml/litre DMSO. The cells were either held at −25°C for 5 min before being immersed in liquid nitrogen or were immersed directly. One week later, the cells were thawed and tested by the schizont-infected cell agglutination test. Variant-specific agglutination was readily demonstrated with cells that had been plunged directly into liquid nitrogen. This indicated that a high proportion of the thawed infected red cells retained their specific antigenic characteristics. Red cells cooled by the two-step method, however, showed considerable nonspecific agglutination.

In a similar experiment (Fig. 4), schizonts were cooled in an equal volume of physiological saline solution containing 38 g of glycerol and 2.9 g of sorbitol per 100 ml. In this instance, the two-step procedure involved a 15-min holding period at −32°C before immersion in liquid nitrogen. Again, the agglutination titres obtained with cells plunged directly into liquid nitrogen were comparable with

Fig. 5. Tissue culture of ring-stage parasites of \textit{P. knowlesi} preserved by direct cooling to −196°C or by rapid cooling from a holding temperature of −30°C. (A) Percentage haemolysis on thawing. (B) Number of parasites in tissue culture. (C) Incorporation of \textsuperscript{3}H-isoleucine by growth of rings to trophozoites in tissue culture. P = plunged; NOP = not plunged.

Fig. 6. Electron micrographs of freeze-substituted trophozoites of \textit{P. knowlesi} cooled either (A) directly to −196°C or (B) to −196°C from a holding temperature of −25°C. Numerous ice cavities (arrows) were present in parasites plunged to −196°C, but cells prepared by two-step cooling were grossly shrunken and contained relatively little intracellular ice. The scale bar represents 1 μm.
those of unfrozen cells, whereas cells stored by the two-step procedure were agglutinated nonspecifically.

Freezing of ring-stage parasites

Washed blood containing 10% infected red cells (84% of the parasites were rings) was mixed with an equal volume of 200-ml/litre DMSO in Ringer's solution. Duplicate samples were held at −30°C for 10, 30, or 45 min before one tube from each pair was plunged into liquid nitrogen. Thawed, reconstituted cells were grown in tissue culture for 17 h; during this time the parasites grew from ring stages to large trophozoites. As shown in Fig. 5, there was no evidence that the two-step procedure provided any extra protection of ring-stage parasites compared with rapid plunging. In three similar experiments, it was found that 25–50% of the frozen and thawed rings survived and grew to trophozoites and schizonts with very little alteration in morphology, even after direct immersion in liquid nitrogen.

Freeze substitution

Electron micrographs of freeze-substituted trophozoites and schizonts of P. knowlesi preserved by direct cooling to −196°C or by the two-step method are shown in Fig. 6. Parasites cooled rapidly to −196°C had ice crystal cavities distributed throughout the cytoplasm and nuclei. Large cavities occurred in some cells (arrow in Fig. 6A) but numerous small ice crystals formed in the majority. There were very few cells without intracellular ice. Cells held at −25°C for 5 min before being cooled to −196°C were, by contrast, severely shrunken and contained relatively few ice crystal cavities. Intracellular ice was not uniformly distributed in ring-stage parasites (not shown) that had been plunged directly into liquid nitrogen.

DISCUSSION

Two-step cooling procedures have been found to improve protection against freezing and thawing injury with several different types of cell (4). Protection results from the formation of extracellular ice during cooling, which produces an osmotic gradient that leads to dehydration and shrinkage of cells at the higher holding temperature. This minimizes intracellular ice damage during rapid thawing from the low storage temperatures.

Electron micrographs of freeze-substituted trophozoites and schizonts of P. knowlesi showed that cells preserved by the two-step method were grossly shrunken and contained relatively small amounts of intracellular ice. This was not the case with cells that had been rapidly cooled to −196°C. Ring-stage parasites that had been plunged directly into liquid nitrogen were more heterogeneous in appearance, some cells having more intracellular ice than others. Further work is needed to find the best method of reducing intracellular ice formation during cooling and also to relate the amount of ice to cell survival on thawing.

The literature on freezing and thawing intra-erythrocytic malarial parasites lacks a systematic study of all the factors involved. In the earliest studies (reviewed by Smith, 10), whole infected blood was frozen and thawed in the absence of cryoprotectants. Survival of P. berghei was improved by the addition of glycerol as a cryoprotectant (11). DMSO was found to be an alternative cryoprotectant but the survival of P. berghei cooled at 2°C per min to −20°C in 70-ml/litre DMSO, and then rapidly thawed, was estimated to be only 1–3% (12).

A two-stage cooling procedure was used by Booden & Geiman (13) to preserve P. knowlesi and other primate malarial parasites. Blood in 75-ml/litre DMSO was cooled at 1–2°C per min to −25°C. After they had been held at this temperature for 1 h, the samples were plunged into liquid nitrogen. Rapidly thawed blood was shown to be highly infective but the extent of parasite survival was not measured. A negligible loss of parasite viability was claimed by Pavanand et al. (14). In their experiments, washed erythrocytes infected with ring stages of P. falciparum were cooled directly to −196°C in the presence of 80-, 120-, or 150-ml/litre DMSO. Cells rapidly frozen and thawed in 120-ml/litre DMSO showed the least lysis. Some damaged parasites were observed but most underwent schizogony and multiplied in vitro like the unfrozen controls. It was estimated by Haynes et al. (15) that 20–50% of ring-stage parasites of P. falciparum survived freezing and thawing by the method of Meryman & Hornblower (16) for the preservation of red cells in 6.2-mol/litre glycerol.

Our studies of frozen and thawed rings, trophozoites, and schizonts of P. knowlesi indicate that there is a lack of correlation between the survival of red cells and parasites. Rapid cooling to −196°C was best for the preservation of erythrocytes and ring forms of the parasite; this procedure, however, almost totally destroyed large trophozoites and schizonts. It may indeed provide a method for
selective destruction of unwanted stages of the parasite; for example, to improve synchrony. Preservation of the late developmental stages of the intraerythrocytic parasite was, on the other hand, greatly improved by two-step cooling to \(-196^\circ C\). This procedure altered the surface of the erythrocytes so that they tended to agglutinate spontaneously, a difficulty that did not occur with cells plunged directly into liquid nitrogen. Consequently, only the latter method was suitable for the storage of antigenically active (but largely nonviable) schizont-infected cells for use in agglutination tests.

It seems likely that two-step cooling procedures will prove best for the cryopreservation of viable forms of large intracellular stages of other species of malarial parasite, whereas direct immersion in liquid nitrogen will be best for ring-stage parasites. It needs to be stressed, however, that particular cooling and thawing protocols should not be applied indiscriminately from one system to another. To determine the best cooling and thawing conditions, the two-step procedure that we have described is recommended as a simple analytical method that can be applied to other host–parasite systems.

RÉSUMÉ

CONSERVATION DE FORMES INTRA-ÉRYTHROCYTAIRES DES PARASITES DU PALUDISME PAR DES PROCÉDÉS DE REFROIDISSEMENT EN UN TEMPS ET EN DEUX TEMPS

On a refroidi dans du diméthylsulfoxide des formes annulaires, des stades trophozoïtes et schizontes de Plasmodium knowlesi, soit en les plongeant directement dans de l'azote liquide, soit par une méthode en deux temps qui consistait à tenir les cellules à des températures inférieures à zéro mais pas trop basses pendant des temps variés avant de les congeler à \(-196^\circ C\). Après le traitement direct par l'azote liquide, les trophozoïtes et les schizontes décongelés se sont révélés fortement endommagés. Leur survie était notablement augmentée lorsqu'on tenait les cellules à \(-31^\circ C\) pendant 30 minutes avant de les plonger dans l'azote liquide. La cryosubstitution montrait que les cellules réfrigérées par la méthode en deux temps étaient fortement rétractées et présentaient relativement peu de cavités intracellulaires dues à la glace. De grandes quantités de cristaux de glace étaient formés dans les trophozoïtes et les schizontes conservés par immersion directe dans l'azote liquide. Les protocoles en deux temps qui ont été étudiés n'amélioraient pas la survie des formes annulaires du parasite dont 25 à 50% survivraient à la congélation rapide à \(-196^\circ C\). Des épreuves d'agglutination ont été effectuées sur des globules infectés de schizontes congelés et décongelés. Une spécificité de variant a été démontrée dans le cas des cellules qui ont été portées directement à \(-196^\circ C\), mais celles qui ont été refroidies par la méthode en deux temps tendaient à s'agglutiner spontanément.

REFERENCES