

Long-term storage of blood in liquid nitrogen, and the response of the recovered red cells to haemagglutination by viruses

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SUMMARY Human and chicken blood were snap-frozen as droplets in liquid nitrogen. Storage in the vapour phase liquid nitrogen container for nine months followed by rapid thawing resulted in approximately 10% haemolysis of the red cells. The ability of the thawed red cells to haemagglutinate viruses was unimpaired by the manipulations.

The initial procedure for long-term storage of red blood cells was in 15% glycerol at -79°C^1 or 30% glycerol and 3% citrate at -20°C^2 . This did not adequately maintain the integrity, enzymes, and antigens of such erythrocytes, and the presence of glycerol induced the formation of glutinous masses which resisted resuspension.³ These problems were largely overcome by the introduction of the high-glycerol, slow-freeze,⁴ and low-glycerol, fast-freeze⁵ methods now in routine use in some blood banks. Both techniques involve the removal of the glycerol by serial or continuous flow centrifugation. Huntsman and colleagues^{3, 6} introduced a method elaborated by other workers^{7, 8} for storage in liquid nitrogen of blood cells in droplet form which had little or no adverse effect on the blood grouping or typing qualities.

This report extends the technique to snap-freezing in liquid nitrogen of whole human and chicken blood, which can then be stored in the vapour phase, and deals with the reaction of such thawed, washed, red cells to haemagglutination by selected viruses.

Material and methods

Samples of whole human and chicken blood were collected, and clotting was prevented by adding 1 mg per ml ethylenediaminetetraacetic acid (EDTA). To each blood sample was added half its original volume of sterile 40% (w/v) sucrose solution as an extracellular cryoprotective. The technique of Huntsman *et al*^{3, 6} was used to freeze the blood. The volume of the drops and the distance each had to

fall to the liquid nitrogen were critical, 25 μl and 75 cm being optimal. The ball bearings of frozen blood, 2 mm in diameter, were removed from the liquid nitrogen by pouring it through a fine wire screen, deposited into precooled, suitably labelled, glass screw-capped vials, and subsequently placed in a vapour-phase liquid nitrogen cabinet at a maximum temperature of -148°C .

When required, a suitable number of pellets amounting to a known volume of blood was removed with forceps, the ends of which had been cooled in liquid nitrogen. They were immediately placed in 10 times their own volumes of phosphate buffered saline⁹ at 40°C . These were shaken to encourage thawing, which took a few seconds, and the blood was centrifuged in an MSE Coolspin at 1000 *g* for 10 minutes to sediment intact cells. The supernatant fluids contained haemoglobin released by the freeze-thaw process. The pelleted cells were lysed by topping up to the original volume with distilled water followed by 10 minutes' ultrasonication in an EM-Scope bath type ultrasonicator. The haemoglobin solutions before and after lysis of blood samples were centrifuged at 4000 *g* to remove the stroma, and the supernates were diluted to the same degree with Isoton 11.* The haemoglobin present in 10 ml of each solution was converted to cyanmethaemoglobin by the addition of 3 drops Zapoglobin* for reading on a haemoglobinometer* at a wavelength of 540 nm. The haemolytic action of the freezing procedure was determined as a percentage of the total haemoglobin in each specimen. Measurements were made in

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duplicate on blood from each species removed from the liquid nitrogen store at three-monthly intervals.

Samples of unlysed washed red cells were prepared as 0.25% (v/v) suspensions in phosphate buffered saline and used in routine haemagglutination tests using 25 µl volumes¹⁰ against serial twofold dilutions of haemagglutinin from influenza virus A strains USSR/90/77, Texas/1/77, Victoria/3/75, and Scotland/840/74, influenza virus B strain Hong Kong/8/73, parainfluenza virus types 1 and 3, and Sendai virus (Division of Microbiological Reagents and Quality Control, Colindale). Comparative tests with red blood cells from each species before and after the freezing process were done.

Results and discussion

The recovery of intact cells over a nine-month period, as measured by the amount of haemolysis induced by the freeze-thawing procedure, averaged 93.2% for human and 90.4% for chicken cells (Table 1). The initial freezing process followed by

Table 1 *Haemolysis due to freezing and storage of whole blood in liquid nitrogen*

Blood	Storage period (months)		
	3	6	9
Human 'O'	94.2*	91.6	93.7
Chicken	89.3	91.2	90.8

*Concentration of haemoglobin in cells recovered intact expressed as % of total haemoglobin in sample.

rapid thawing results in a loss of approximately 10% of cells irrespective of intervening storage time, provided the low temperatures are maintained. Under these conditions the cooling and thawing rates were each approximately 1500°C per minute. After freeze-thawing, the washed red cells lysed more rapidly than fresh blood over three or four days' storage at +4°C and should therefore be used as soon as possible after reconstitution.

The mechanism of this lysis is unknown, but changes in the cell wall due to dissociation and denaturation of lipoproteins resulting in osmotic fragility are probable. Ultrastructural studies by thin sectioning, freeze-etching, and scanning electron microscopy on fresh and freeze-thawed red cells may resolve this.

The established method for long-term storage in liquid nitrogen of cells for culture use in virology laboratories with di-methyl sulphoxide as a cryoprotective has been described.¹¹ The temperature of the cells was lowered by 1°C per minute until the critical temperature of -65°C was reached. If

this was done too rapidly excessive cell loss occurred. Nonetheless cell recovery of 50-70% of the original concentration is considered average. However, freezing rates of 100 to 300°C per minute¹² and two-stage freezing procedures¹³ have resulted in satisfactory survival of selected mammalian cell types. The methods used for the cryopreservation of human tissues, leucocytes, platelets, and some cell lines have been reviewed.^{8 14}

It remains to be seen whether the technique now reported could be used for cell culture lines in a diagnostic virology or cytology laboratory. Should this prove so, it has the advantage of simplicity, requires no careful cooling procedures, and has the potential for high cell yields after long-term preservation.

The ability of the freeze-thawed, washed red cells to haemagglutinate the eight virus strains tested was unaffected, and the titres against both species of red blood cells before and after freezing were similar (Table 2). This means that any changes that occur in the cell wall due to this process do not affect the haemagglutinin receptor sites on the red cells.

Table 2 *Ability of freeze-thawed red cells to haemagglutinate viruses*

Virus	Human 'O' cells		Chicken cells	
	Fresh	Freeze-thawed	Fresh	Freeze-thawed
Influenza A				
USSR/90/77	256*	256	128	64
Texas/1/77	128	128	64	64
Victoria/3/75	512	512	512	512
Scotland/840/74	512	1024	256	512
Influenza B				
Hong Kong/8/73	128	128	256	256
Parainfluenza 1	64	64	<4	<4
Parainfluenza 3	32	16	<4	<4
Sendai virus	2048	2048	2048	2048

*Reciprocal of virus dilution, average of two tests.

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