Cryopreservation of human granulocytes in liquid nitrogen

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SUMMARY Human granulocytes (PMNL) were successfully cryopreserved for up to 14 months. The PMNL ($1-2 \times 10^7/\text{ml}$) were stored in 2-ml ampoules in the gas phase of liquid nitrogen at a temperature between -160°C and -196°C using dimethylsulphoxide (DMSO 10%) as cryoprotectant. Morphology and phagocytic and bactericidal capacity were best preserved by adding fetal calf serum to the freezing mixture, by using an interrupted cooling process, by washing the thawed PMNL in fresh freeze-dried plasma, and centrifuging at 600 g for no more than two minutes. Careful post-thaw handling of the cells was an important factor in preserving function. These preliminary studies indicate that useful numbers of PMNL can be recovered in a functional state after storage for long periods in liquid nitrogen.

Attempts to preserve mature polymorphonuclear leucocytes (PMNL) for long periods at low temperatures have met with little success (Cavins *et al.*, 1968; Crowley *et al.*, 1974; Pegg, 1972). PMNL are more susceptible to cooling and freezing injury than other blood cells (Pegg, 1972), damage apparently resulting from thermal shock during cooling, osmotic changes during freezing and thawing, and intracellular ice formation (Knight *et al.*, 1975). The widespread use of white cell transfusions in leukaemic patients during periods of marrow depression, however, has created a need for readily available PMNL and makes the development of 'cell banks' a desirable aim. A recent report of PMNL storage at -80°C for up to three months has given hope that these cells might be cryopreserved in a viable state (Lionetti *et al.*, 1975). We describe here our experience with storing PMNL in the gas phase of liquid nitrogen at a temperature between -160°C and -196°C (in liquid nitrogen vapour at -196°C) with recovery of functional cells after cryopreservation for up to 14 months.

Material and methods

All PMNL batches were collected into 300-ml

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sterile bags containing 25 ml ACD (formula A) during leucapheresis of normal donors with an Aminco cell separator (American Instrument Company, Maryland, USA). Aliquots of PMNL comprising equal volumes of cells (usually $1-2 \times 10^7/\text{ml}$) and freezing mixture were placed in 2-ml ampoules. The mixture consisted of two parts of tissue culture medium (TC 199), two parts of fetal calf serum (FCS), and one part of dimethylsulphoxide (DMSO). The total WBC concentration was determined by a Coulter electronic counter (model FN) and the proportion of PMNL by Leishman-stained smears. The ampoules were flame-sealed and then frozen in an inverted wire basket using a low temperature refrigeration unit (G. V. Planer Ltd) before being stored in the vapour phase of a liquid nitrogen refrigerator (Union Carbide Ltd). An interrupted cooling procedure was found better than a continuous one, the cooling rate being controlled by temperature-sensing devices according to a pre-arranged programme. 'Continuous' cooling consisted of cooling at 1°C per minute from $+20^\circ\text{C}$ to about -60°C then placing the cells directly into liquid nitrogen. 'Interrupted' cooling consisted of cooling at 1°C per minute to -20°C , holding the cells at this temperature for about 15 minutes, then resuming cooling at 1°C per minute to -60°C before transferring to the liquid nitrogen refrigerator. The PMNL were stored from 1 to 14 months.

Cell morphology and function were assessed after rapid thawing in a water bath at 37°C before and

after washing twice in fresh freeze-dried plasma (FFDP) with centrifugation at 600 *g* for two minutes. Several other media for washing the cells were abandoned because of reduction in viability. Slower centrifuge speeds failed to concentrate the cells adequately while longer periods of centrifugation resulted in cell damage. The working temperature for handling the thawed PMNL was +4°C.

The morphology of the PMNL was assessed using Leishman-stained smears, and changes were graded by criteria similar to those of McCullough *et al.* (1974). Slight changes included loss of nuclear chromatin detail, vacuolation, and reduction in cytoplasm. Moderate changes were further loss of nuclear detail and hyperchromia. Advanced changes indicated cells barely recognisable as PMNL.

The function of the cryopreserved PMNL was compared with freshly drawn PMNL using tests of phagocytic and bactericidal capacity.

Phagocytosis was tested by the method of Miller (1969). Baker's yeast adjusted with Hank's buffered salt solution (BSS) to a concentration of 10^9 particles/ml plus freshly thawed AB serum were incubated with PMNL at a concentration of 5×10^6 ml in plain plastic tubes (in a proportion of 0.2 ml cells, 0.1 ml yeast suspension, 0.1 ml serum) for 30 minutes at 37°C with constant rotation. After centrifuging at 500 rpm for four minutes smears of the sediment were Leishman-stained and the percentage of PMNL containing yeast particles counted.

Bactericidal capacity was tested by the method of Solberg and Hellum (1972). 0.5 ml of PMNL suspension (10^7 /ml) were incubated with 0.1 ml bacterial suspension (10^8 /ml) from a standard strain of *Staphylococcus aureus* (Oxford Heatley Strain,

National collection of type cultures, Colindale) plus 0.4 ml BSS with added AB serum at +37°C for two hours with constant rotation. Samples were collected at zero, 7½, 15, 45, 90, and 120 minutes.

Both the total number of viable bacteria and the number of intracellular viable bacteria were determined. The total number was estimated after adding 0.01 ml of PMNL-bacteria suspension to 1 ml distilled water to disrupt the cells and plating out by a standard pour-plate technique. The intracellular bacteria remaining viable in each sample were counted in a similar way, but the PMNL-bacteria suspension was first incubated in BSS containing streptomycin and penicillin before disrupting the cells with distilled water (Solberg and Hellum, 1972). Fresh control PMNL were tested in parallel and a total of eight pairs of stored and fresh cells were analysed.

Results

A total of 19 batches of cells were analysed after storage from 1 to 14 months.

PMNL COUNTS

Satisfactory PMNL counts were obtained in all tests. The mean cell count after thawing and washing twice was 5.3×10^6 PMNL, representing an average loss of 50-75% from the original numbers frozen. By far the greatest source of cell loss was the washing procedure, a 47% reduction in PMNL count following centrifugation at 600 *g* for two minutes, which was found to be the optimal centrifuge procedure.

MORPHOLOGY

All the batches suffered some injury, but most

Fig. 1 Morphological appearances of polymorphonuclear leucocytes stored in liquid nitrogen vapour at -196°C for six months. (Leishman stain $\times 100$)

Fig. 2 Phagocytosis of yeast particles by the same cells shown in Fig. 1. (Leishman stain $\times 100$)

Fig. 3 Total number of bacteria remaining viable during incubation with fresh and cryopreserved PMNL (mean ± 1 SD)

Fig. 4 Number of intracellular bacteria remaining viable during incubation with fresh and cryopreserved PMNL (mean ± 1 SD)

contained about 50% PMNL with only mild changes regardless of the period of storage (Fig. 1). Morphology was improved by (a) the interrupted cooling process; (b) adding FCS to the freezing suspension; (c) washing in FFDP rather than TC199 or buffered saline; and (d) centrifuging for no more than two minutes at high *g*.

PHAGOCYTOSIS

The proportion of actively phagocytic PMNL varied widely under different conditions and we did not attempt to compare actual numbers of particles ingested.

Changes in phagocytic capacity tended to parallel morphological changes, however, about two-thirds of the cells in any batch being phagocytic under the conditions found optimal for morphological preservation (Fig. 2). Storage time again did not affect the results, means of 65% and 60% phagocytic cells being present in batches stored for more and less than four months respectively. 'Interrupted' was better than 'continuous' cooling (means: 70% and 58% phagocytic respectively). Washing with media other than FFDP or centrifugation for longer than two minutes reduced phagocytosis.

BACTERICIDAL CAPACITY

Eight batches of stored PMNL were compared with fresh cells for total and intracellular killing capacity (Figs. 3, 4). These were selected on the basis of a high percentage of viable cells (mild morphological changes and more than 75% cells actively phagocytic). The proportion of total and intracellular bacteria killed by the stored cells after 120 minutes' incubation compared favourably with the fresh cells. Of the total bacteria present initially 11% and 10% remained viable after 120 minutes' incubation with fresh and cryopreserved PMNL respectively; 13% and 27% of the initial intracellular bacteria were viable after 120 minutes' incubation with fresh and cryopreserved PMNL respectively.

Discussion

Storage of mature PMNL at +4°C results in increasing loss of function after the first 24 hours (McCullough *et al.*, 1974), and lower temperatures are needed wholly to arrest cell metabolism. Successful preservation in liquid nitrogen of actively phagocytic PMNL was reported more than a decade ago (Cavins *et al.*, 1965; Rowe and Cohen, 1965), though early transfusion experiments showed these cells to retain only about one-fiftieth of the efficiency of fresh PMNL (Roy and Djerassi, 1969). Interest in their preservation revived when improved leucapheresis techniques facilitated large PMNL yields

from normal donors (Boggs, 1974; *Lancet*, 1975). Nevertheless, several studies using both -80°C and -196°C as storage temperatures failed to improve on the early results (Skeel *et al.*, 1969; Malinin, 1972; Crowley *et al.*, 1974), PMNL viability being measured by basic dye exclusion, motility, phagocytosis, and different enzymatic functions.

The causes of failure have been carefully analysed by Knight *et al.* (1975). Thermal shock before the freezing temperature is reached can be prevented by slow cooling at 0.3°C per minute. Though continued slow cooling may allow sufficient cell shrinkage to prevent intracellular ice formation, it also exposes the PMNL for a longer period to adverse osmotic conditions. Since the freezing/thawing process is damaging, both from osmotic changes and from intracellular ice formation, a protective effect has been achieved by cooling more rapidly but interrupting the process at -26°C (Farrant *et al.*, 1974; Knight *et al.*, 1975). This lessens the time of exposure to changes in osmolality but also allows cell shrinkage to prevent ice formation.

The greatest risk to PMNL viability, however, may be from sensitisation during freezing to damaging stresses after thawing, because they are probably most sensitive to swelling and to dilution, both of which occur at this time (Pegg, 1972; Farrant, 1975). The post-thaw handling of the PMNL may therefore be the single most important factor in preserving function.

Our results show that functional mature PMNL can be preserved for long periods in liquid nitrogen. Using standard phagocytosis and bactericidal assays we tested those functions most relevant to clinical needs, though we have not attempted to assess the ability of stored cells to circulate *in vivo* and migrate to inflammatory sites.

The adoption of a two-phase cooling process, with a steady rate of cooling from +20°C to -60°C interrupted for a period of adaptation at -20°C, together with the use of fetal calf serum and 10% DMSO in the freezing mixture seemed beneficial. The success of our technique, however, may be related particularly to careful handling after the cells were thawed. We found, like others (Cavins *et al.*, 1968), that phagocytosis was inhibited by the presence of DMSO and that unless homologous plasma rather than other media was used to remove it the cells were irreversibly damaged. Centrifugation at 600 *g* for longer than two minutes was similarly harmful, while keeping the thawed cells at a working temperature of +4°C was beneficial. The effect of these stresses clearly shows the vulnerability of thawed PMNL, which is presumably due to sensitisation during freezing (Farrant, 1975).

The mean PMNL recovery after DMSO removal

was 5.3×10^6 per ampoule (less than 50% of starting value), of which not all retained good morphology and function. Even assuming they proved bactericidal *in vivo* and that a useful clinical effect was produced by transfusing 10^{10} stored PMNL, it would clearly be impractical to provide the necessary 2000 ampoules every transfusion. We have found in preliminary studies no significant functional differences between PMNL stored in 2-, 10-, and 20-ml batches.

Before transfusion of cryopreserved PMNL could be developed clinically it would be essential to test further the relationship between increasing cell volumes and concentrations and post-thaw viability.

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