Low Temperature Preservation of Mouse Lymphocytes with Dimethyl Sulfoxide

By M. J. Ashwood-Smith

DIMETHYL SULFOXIDE was first introduced as a low temperature preservative agent for mammalian cells by Lovelock and Bishop. Since that time a variety of cells have been successfully stored at low temperatures with this chemical. Ashwood-Smith found dimethyl sulfoxide to be superior to glycerol for the preservation of mouse bone marrow, and Cavins et al. have reported essentially similar findings with dog bone marrow. Glycerol was found to be inferior to dimethyl sulfoxide for the preservation of human cells from tissue culture and recent studies with rabbit corneal tissue have indicated the possible usefulness of dimethyl sulfoxide in the low temperature storage of corneas prior to keratoplasty.

Results with preserved mouse marrow (Ashwood-Smith) indicated that many small and medium lymphocytes present in marrow successfully survived freezing to, storage at, and thawing from -79°C. The recovery of mice which had received lethal doses of whole body radiation was excellent when preserved marrow was given immediately after x-irradiation. Lymphoid tissue of these mice recovered and there was no doubt that dimethyl sulfoxide had protected a sufficient number of lymphocytes and possibly lymphoid stem cells from the damaging effects of freezing and thawing. This present study has been undertaken in an attempt to ascertain the best parameters for the preservation of lymphocytes which are normally found in lymph nodes.

One of the greatest difficulties associated with low temperature biology is to develop a method or a series of methods which will give the best possible answer to the question of assessing cell viability after freezing. In this study several different methods for assaying viability have been used. The morphology, motility and biochemistry of fresh and preserved lymphocytes have been studied by in vitro methods. The biological effectiveness of preserved lymphocytes has been established by using parental strain lymphocytes which have the property of eliciting a graft versus host reaction when injected into F1 hybrids. The basis of this reaction has been discussed by Simonsen, and the resulting splenomegaly induced in the host by the action of the injected lymphocytes can be used, within limits, to assay the viability of the injected cells. The splenomegaly is largely a result of host cell proliferation and is partly abolished by prior irradiation of the host. It has been shown that this splenomegaly can be induced in adult mice if a sufficient number of cells are given. Although sublethal x-irradiation of the adult host still permits a measurable increase in spleen weight to occur, a far larger proportion of the spleen cells are of donor origin under these circumstances. Also, a smaller number

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of lymphocytes are required to elicit the splenomegaly when sublethally x-irradiated adults are used (Ashwood-Smith, unpublished observations).

METHODS

Cell suspensions. Lymphocytes were obtained by gentle homogenization of the brachial, cervical and inguinal lymph nodes. Cells were suspended in Parker "199" tissue culture fluid containing 10 per cent rabbit serum.

Mice. Lymphocytes were obtained from adult "A" strain female mice. In the experiments designed to test the immunologic effects of fresh and frozen lymphocytes, young (14-16 week old) LAF1 hybrid female mice were used as recipients of the injected "A" strain lymphocytes.

Phase contrast microscopy. Lymphocytes were mounted, examined and photographed by phase contrast using the agar mounting technic described by Ashwood-Smith and Young. Protein synthesis studies. Lymphocytes suspended in Parker "199" were incubated for 1 hour at 37 C. in the presence of 5 µc. DL-Valine-4-C¹⁴ (specific activity 5 mc. per ml.). At the end of this time the proteins were extracted with trichloroacetic acid and analyzed for radioactivity. All radioactivity measurements were expressed as counts/min. of protein (dry weight).

Freezing procedures. A Linde BF₃ liquid nitrogen freezing apparatus was used for freezing cell samples. This machine enables controlled rates of cooling between 1 C./min. and 19 C./min. to be obtained. In most experiments a cooling rate of 4 C./min. was used until the cooling rate of the sample slowed up as the heat of crystallization occurred. At this point (usually -10 C.) the rate of cooling was increased to give the maximum rate of 19 C./min. Preliminary experiments with faster cooling rates failed to indicate any advantages over the standard rate of 4 C./min. Ampules were then transferred to a Linde LNR25 liquid nitrogen refrigerator for storage.

All samples were thawed rapidly by immersing the ampules in water at 40 C. For biochemical and morphologic examination the concentrations of dimethyl sulfoxide in the thawed cell samples were reduced to about 1 per cent by the slow addition of fresh tissue culture fluid. For the in vivo determinations of viability the glycerol or dimethyl sulfoxide concentrations were not reduced before injection of the cells into mice.

X-Irradiation. A Westinghouse 250 kvp x-ray machine was used for irradiating the "LAF1" mice. The following irradiation factors were used: 600 rads of x-rays delivered at a dose rate of 30 rads/ min.; 250 kvp, 15 ma., 0.5 mm. copper and 1.0 mm. of aluminum filtration; HVL of 1.28.

RESULTS

Morphology and motility of fresh and frozen lymphocytes. Phase contrast photographs of normal, fresh lymphocytes are shown in figure 1 (A, B). Over 80 per cent of the cells in a freshly made preparation of lymph node lymphocytes were found to be intact and to display normal motility when mounted on agar slides and observed at 37 C. Lymphocytes which had been frozen to -196 C. without the benefit of protective agents were destroyed. The majority of the frozen cells had been stripped of their cytoplasm and the naked nuclei were often clumped together. Occasionally a few cells were seen immediately after freezing and thawing in which the cytoplasm was still intact but badly vacuolated (fig. 1, C). Further observation of these cells revealed that this vacuolation was followed within minutes by complete disruption of the cytoplasm.

When lymphocytes were frozen in the presence of dimethyl sulfoxide, many cells appeared intact and moved normally. The optimum concentration of
dimethyl sulfoxide was 15 per cent and a sample of lymphocytes which had been frozen to \(-196\, ^\circ\mathrm{C}\) in the presence of 15 per cent dimethyl sulfoxide and then thawed is shown in figure 1 (D). Concentrations higher than 15 per cent and lower than 7.5 per cent gave very little protection.

Protein synthesis in vitro. Fresh lymphocytes incorporated radioactive valine readily and at the end of 1 hour of incubation the radioactivity measurements were usually in the region of 1000 to 2000 counts/min. of dry protein. Cells which had been deliberately damaged by homogenization synthesized little if any protein. Lymphocytes which had been frozen to \(-196\, ^\circ\mathrm{C}\) and thawed in the absence of dimethyl sulfoxide were also virtually devoid of protein synthesizing activity. Results of these experiments are shown in table 1.

These results were very similar to those obtained by Ashwood-Smith with mouse bone marrow cells.\(^{2,10}\) In order to test the ability of frozen lymphocytes...
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Table 1.—The Incorporation of DL-Valine-4-C\textsuperscript{14} by Mouse Lymphocytes in Vitro into Protein

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weight of Isolated Protein (mg, dry weight)</th>
<th>Counts/Minute</th>
<th>Specific Activity Counts/Minute/mg. of Protein (dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.40</td>
<td>680</td>
<td>1700</td>
</tr>
<tr>
<td>Homogenized cells</td>
<td>0.40</td>
<td>34</td>
<td>85</td>
</tr>
<tr>
<td>Frozen cells (no protective agent)</td>
<td>0.50</td>
<td>36</td>
<td>72</td>
</tr>
</tbody>
</table>

Duplicate 1 ml. samples. Parker "199" tissue culture fluid containing 40 x 10\textsuperscript{6} cells and 5 \mu c. of valine-4-C\textsuperscript{14}. Incubation for 1 hour at 37 C.

to synthesize proteins after freezing and thawing in the presence of dimethyl sulfoxide, it was necessary first to reduce the concentration of dimethyl sulfoxide in the thawed cells to below 5 per cent. At concentrations greater than this, the compound has a marked inhibitory action on the protein and lipid synthesis by mouse marrow cells;\textsuperscript{10} this inhibitory effect is, however, reversible. Lymphocytes suspended in Parker "199" containing different amounts of added dimethyl sulfoxide were frozen to \(-196\) C. and held at that temperature for 5 hours. They were then thawed rapidly and assayed for protein synthesizing activity. The results of this experiment are shown in figure 2, and are expressed as percentages of the radioactivity count for the fresh, unfrozen lymphocyte sample. Cells frozen in the presence of 10 per cent dimethyl sulfoxide retained about 50 per cent of their protein synthesizing ability. At concentrations of sulfoxide higher than 15 per cent, biochemical activity was considerably reduced.

Fig. 2.—Protein synthesis of fresh and frozen mouse lymphocytes (incorporation of DL-valine-4-C\textsuperscript{14} into proteins during in vitro incubation).
Biological assessment of fresh and frozen lymphocytes. The splenomegaly produced in adult LAF₁ mice by the injection of lymphocytes from adult "A" strain mice was used to assess the viability of frozen lymphocytes. The recipient mice were given a sublethal dose (600 rads) of x-rays 18 hours before the intraperitoneal injection of the lymphocytes. Sublethal irradiation reduces the number of lymphocytes required to induce the increase in spleen size. Mice were killed 7 days after the cell injections and the paired inguinal lymph nodes and the spleens were weighed.

The increase in spleen weight is roughly proportional to the number of injected lymphocytes, although when more than 10 X 10⁶ lymphocytes are injected the splenomegaly does not increase but reaches an optimum. The weight of the inguinal lymph nodes follows a similar but more variable pattern.

The spleen response after injection of fresh and frozen lymphocyte. Groups of 10 mice were sublethally x-irradiated and then injected with 5.4 X 10⁶ parental strain lymphocytes. One group received no lymphocytes (control) and the other groups received 5.4 X 10⁶ lymphocytes which had been frozen to -196 C. and then thawed 2 hours later; lymphocytes were frozen in the absence of any protective agent, in the presence of 10 per cent or 15 per cent dimethyl sulfoxide, and in the presence of 10 per cent or 15 per cent glycerol. The results of this experiment are shown in figure 3; results are expressed in terms of spleen or lymph node weight (mg./100 Gm. mouse ± standard deviation). It is apparent that cells frozen in the absence of any protective agent are virtually ineffective in producing splenomegaly. Cells frozen with glycerol as a preservative show some biological activity but the best results were obtained with cells frozen in the presence of 15 per cent dimethyl sulfoxide when the extent of the lymph node and spleen enlargement was almost as great as that achieved with the same dose of fresh cells.

The spleen response after injection of lymphocytes which had been preserved for 3 months at -196 C. Lymphocytes preserved in 10 per cent and 15 per cent dimethyl sulfoxide were frozen at the normal cooling rate and kept at -196 C. for 3 months, at which time they were injected into sublethally x-irradiated mice. Two standard doses of fresh lymphocytes were used, namely 7.6 X 10⁶ and 3.8 X 10⁶ in two groups of mice. Another two groups of mice were injected with that volume of the cell suspension which should have contained 7.6 X 10⁶ cells had all the lymphocytes survived the period of storage. Another group of mice received no cells and a last group received 7.6 X 10⁶ cells which had been frozen without the benefit of any protective agent. Results are shown in figure 4, and it is apparent that the cells preserved in the presence of 15 per cent dimethyl sulfoxide were capable of eliciting a splenomegaly greater than that produced by half that number of fresh cells but not as large as that produced by injection of the same number of fresh lymphocytes. It can be concluded, therefore, that more than 50 per cent of those cells responsible for causing a graft versus host reaction, probably the small lymphocytes, survived storage at -196 C. in the presence of 15 per cent dimethyl sulfoxide.

Discussion

The morphologic evidence and the results of the motility experiments indicate that 15 per cent dimethyl sulfoxide gives better protection than 10 per
Fig. 3.—Spleen and lymph node weights in sublethally x-irradiated LAF₁ (female) mice injected with fresh and frozen lymphocytes ("A" mice female); 5.4 x 10⁶ cells per mouse.

cent sulfoxide and this agrees well with the results obtained with the splenomegaly assay. The biochemical data suggest that 10 per cent sulfoxide is the optimum amount of sulfoxide for protective action and this result is at variance with the answers given by the other test systems. It is highly probable that this difference is an artifact as the concentration of sulfoxide must be reduced to levels below 5 per cent before the biochemical assay can be started. The higher the original concentration of sulfoxide, then the greater is the possibility of some osmotic damage occurring prior to the incubation with the radioactive
Fig. 4.—Spleen and lymph node weights in sublethally x-irradiated LAF₁ (female) mice injected with fresh lymphocytes and lymphocytes ("A" strain female mice) which had been stored for 3 months at −196 C. in the presence of dimethyl sulfoxide.

Amino acid. When cells preserved with dimethyl sulfoxide are injected into mice, the removal of sulfoxide from these cells still takes place but it does so under natural conditions and any nonlethal damage sustained by the cells may well be repairable in vivo but not in vitro.

It is clear that 15 per cent dimethyl sulfoxide is an effective protective agent for mouse lymphocytes and the results reported in this paper are very similar
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to those obtained with mouse and dog bone marrow preservation with
dimethyl sulfoxide. It should not be forgotten, however, that lymph node
lymphocytes are not an homogenous cell population but a mixture of lympho-
cytes of various sizes, plasma cells and monocytes, and the splenomegalaly
assay system is probably only measuring the viability of the small lymphocyte.

Summary

Mouse lymphocytes have been successfully preserved for 3 months at −196
C. in the presence of 15 per cent dimethyl sulfoxide. Other concentrations of
dimethyl sulfoxide or glycerol were less effective. The viability of the frozen
lymphocytes was assessed by phase contrast microscopy, protein synthesizing
activity and by the ability of these lymphocytes to produce splenomegalaly when
injected with F₁ hybrids.

Summary in Interlingua

Lymphocytos murin esseva conservate a bon successo durante tres menses
a un temperatura de −196 C in le presentia de 15 pro cento de sulfoxydo
dimethylic. Altre concentrationes de sulfoxydo dimethylic o de glycerol esseva
minus efficace. Le viabiitate del congelate lymphocytos esseva evalutate per
medio de microscopia a contrasto de phase e a base de lor activitate de
synthese de proteina e lor capacitate de producer splenomegalia post lor in-
jecction in muses hybrida F₁.

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