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Approaches to the Preservation of Human Granulocytes by Freezing[1]

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INTRODUCTION

Although there have been numerous attempts to preserve functional human granulocytes by freezing (1,3,4,5,7,12), most of them have yielded low survivals at best. In the few cases where high functional viabilities have been reported (2,6,11), the reasons for the success are mysterious in that the procedures used in these studies appear to be only slight variations of techniques which others have reported to yield little or no success.

To help explain the puzzling divergence in results and hopefully to help develop procedures that will lead to repeatable successful preservation, we decided to examine the response of human granulocytes to the major cryobiological factors known to determine the survivals of other types of cells after freezing and thawing. Chief among these factors are the type, concentration, and toxicity of cryoprotectant, the cooling and warming rates, and the amount of osmotic stress imposed upon the cells during dilution of the cryoprotectant after thawing. Most previous studies of granulocyte preservation have used dimethyl sulfoxide (Me_2SO) in submolar concentrations as the cryoprotective agent. Since there is evidence that submolar concentrations may not confer complete protection (10) and some evidence that concentrations of Me_2SO above 1 molar can be toxic, we decided to use glycerol instead. There is also some suggestion in the literature that human granulocytes are sensitive to osmotic stress, since slow dilution of the additive has been reported to give higher survival than rapid or abrupt dilution (6). Finally, there is little but anecdotal information on the effects of cooling rate and warming rate on the postthaw viability of these cells.

Our approach in this study was first to examine the suitability of glycerol as a possible cryoprotective agent by characterizing its toxicity to

granulocytes independent of the effects of dilution. Second, we wanted to devise a dilution procedure that would safely remove cells from multimolar concentrations of the additive. Lastly, we wanted to identify the optimum cooling and warming conditions for these cells by studying survival as a function of cooling and warming rates.

Toxicity of Glycerol

An investigation of glycerol toxicity independent of the stress and injury of dilution requires an assay that can be carried out in the presence of the glycerol. We used a fluorescence assay that is based on the conversion of the nonfluorescent compound fluorescein diacetate (FDA) to the fluorescent molecule fluorescein by intracellular esterases. A positive response in this assay indicates plasma membrane integrity and the retention of cytoplasmic esterase activity (4). Briefly, FDA was added to cells suspended in various concentrations of glycerol/phosphate buffered saline (PBS) to give a final FDA concentration of 1.2×10^{-6} M, and aliquots of the cell suspension were then counted on a hemacytometer under FITC fluorescence at various times. Survival is expressed as the percentage of total cells counted that exhibit fluorescence (i.e., $[FDA^{+}/(FDA^{+} + FDA^{-})] \times 100$). The hemacytometer counts also yielded estimates of the fraction of the original cell population that was recoverable after a given treatment. Gencrally recovery was above 50%.

The toxicity of glycerol increased with its concentration and with the exposure temperature. At room temperature, cells suspended in PBS without glycerol showed > 90% survival after 240 min whereas cells in 2 M glycerol showed only 50% survival after 50-min exposure and < 10% after 90 min. The results with 1 M glycerol fell between these extremes. In contrast, at 0°C cell survival remained high: cells in 2 M glycerol held at 0°C now showed

> 90% survival even after 240-min exposure, a result virtually identical to that obtained with control cells in PBS. Parallel experiments using trypan blue dye exclusion as the viability assay showed qualitatively the same effects of glycerol concentration and temperature.

Although there have been prior reports of poor results with glycerol as a cryoprotective agent, few have separated out the possible contribution to injury of osmotic shock accompanying its removal. The loss in viability noted here, however, appears to be the result of true toxicity, since it occurred in the absence of dilution and was accelerated at higher temperatures. Since our results suggested that the toxicity can be reduced or eliminated by holding the granulocytes at 0°C, subsequent studies were carried out at ice bath temperatures. The next problem was whether the glycerol could be removed from the cells without damage.

Dilution from Glycerol

Rather than trying to devise a suitable dilution protocol empirically or using procedures that work with other cell types, we chose to "custom design" a dilution procedure based on the osmotic properties of human granulocytes themselves. This approach has become feasible through the development of a computer program which models the behavior of a cell subjected to various osmotic manipulations (8,9). Utilizing the coupled differential equations that describe solute permeation and the permeability properties of the cell in question, the program can quickly reveal dilution procedures that should, in theory, produce minimal osmotic stress.

The permeability equations require values for the isotonic cell volume, cell surface area to volume ratio, and cell solids to liquid ratio. These values were determined by computer analysis of cell volume distributions

obtained with a Celloscope electronic cell sizer. The permeability coefficient for glycerol, P_{gly}, was estimated by comparing the actual changes in cell volume as a function of time after the addition of hyperosmotic glycerol with a family of curves generated on the computer where P_{gly} varied over a ten-fold range. The value 4×10^{-5} cm/min provided a reasonable match between the actual and theoretical cell responses as judged by eye and was chosen as the value for P_{gly} at 22°C. Technical difficulties encountered in measuring permeation kinetics at 0°C precluded accurate determination of the temperature coefficient for glycerol permeation; however, the observed changes in cell volume with time at both 22°C and 0°C were quite similar, suggesting that the temperature coefficient is small. The value of P_{gly} at 22°C was therefore used in subsequent computations of osmotic response.

A number of different possible dilution procedures were generated with the computer by imposing various restrictions on the maximum cell swelling permitted at each dilution step and by varying the time allowed for glycerol efflux between successive steps. Four procedures, two each for 1 and 2 M glycerol, were selected on the basis of their convenience of implementation, and were tested on granulocytes. We found that survival, based on the FDA assay, was affected by both the glycerol concentration and the "severity" of the dilution scheme. Cells equilibrated for 30 min in 1 M glycerol showed higher survival after dilution than those in 2 M glycerol, and for both concentrations, the dilution procedure involving a greater number of steps resulted in less injury to the cells. All four procedures, however, yielded quite high survivals both immediately after dilution (> 90% with 1 M glycerol and > 75% with 2 M glycerol) and after an additional 90 min of observation (less than 10% further decline).

Freezing and Thawing

The response of human granulocytes to different cooling and warming rates was evaluated using the FDA assay both before and after dilution of the glycerol. Cooling rates ranged from 0.3 to 33°C/min and warming rates ranged from 1.0 to 188°C/min. In all cases, survival was evaluated as soon after treatment as possible and then monitored at several additional time points during the next hour. Since survival remained relatively constant during this additional time at 0°C, we shall report only the results for the first time point.

The results shown in Fig. 1 indicate that human granulocytes respond to cooling and warming rates in a manner similar to that of other mammalian cells. Survival was low at low cooling rates, increased to a maximum at moderate rates, and decreased again at high cooling rates. The major effect of warming rate was to increase the survival at the higher cooling rates, a finding consistent with the idea of rescue from the effects of intracellular ice formation at supraoptimal cooling rates. The data in Fig. 1 show that the optimum cooling rate with 2 M glycerol is near 3°C/min.

Additional studies further support the notion that human granulocytes exhibit a classical response to cooling and warming. For example, also rather typical was the shift of the optimum cooling rate to a lower value with an increase in the concentration of the cryoprotectant from 1 M to 2 M. This shift was somewhat more pronounced with slow warming than with rapid. Increasing the concentration of glycerol to 2 M also gave better overall protection and reduced the dependence of survival on cooling rate by broadening the range of the optimum, especially when combined with rapid warming.

The above results refer to cells that have been completely diluted free of the glycerol after thawing. To estimate how much injury is attributable to

the freeze-thaw process itself, viability was also assayed with FDA immediately after thawing but without dilution. In most cases, survival was higher in the absence of dilution by a relatively fixed proportion, suggesting that the stresses associated with dilution and those associated with freezing and thawing may operate independently of each other. Under optimum conditions, survival of undiluted frozen-thawed cells was 73% while the diluted samples showed 56%. The difference suggests that approximately 25% of frozen-thawed cells are killed by dilution, a finding that agrees well with the figure obtained for dilution in the absence of freezing and thawing.

Higher Order Functional Test

Although the FDA assay is a good screening test for negative results, meaning that cells judged nonviable by this assay would most probably be nonviable by most other criteria, positive FDA results need to be confirmed by a more definitive measure of cell function. One of the more sensitive measures of higher order function in granulocytes is their ability to undergo chemotaxis. To assess this ability, a sample of frozen-thawed-diluted granulocytes showing > 55% survival after treatment (based on the FDA assay) was placed in the upper compartment of a standard chemotaxis test chamber (Boyden Chamber, #B-312, Neuro Probe, Inc., Bethesda, Md.). After appropriate incubation and processing, the filter was analyzed by optical sectioning to determine the extent to which the cells had migrated toward the stimulus in the lower compartment of the chamber. In contrast to the unfrozen control cells which exhibited good migration, the experimental sample showed absolutely no movement into the filter. Based solely on these results, the FDA assay appears to overestimate the functional capacity of human granulocytes after freezing and thawing. However, a major procedural difference between the two

assays is that the cells being examined for chemotactic capacity were incubated for 2 hr at 37°C after treatment, whereas cells for the FDA assay were held at 0°C until counted. Accordingly, we also used the FDA assay to determine the survival of a frozen-thawed-diluted sample that had been placed at 37°C for various times. The FDA results in this case now agreed with those from the chemotaxis assay in that after incubation for 60 min they showed no surviving cells in the frozen-thawed sample.

SUMMARY AND CONCLUSIONS

Because of its simplicity, the FDA assay can be used effectively as a screening test to eliminate procedures and treatments that are damaging to cells; however, as exemplified by the chemotaxis study, additional tests must be performed to confirm any FDA positive results. In this context, a number of conclusions can be drawn from the data presented: (1) Exposure to 1 and 2 M glycerol at room temperature becomes rather damaging to human granulocytes in a matter of a few tens of minutes. Exposure to 2 M glycerol is much the more damaging. Reducing the exposure temperature to 0°C reduces the amount of injury substantially. (2) Human granulocytes respond to freezing and thawing in a manner typical of many mammalian cells in that they exhibit a maximum in survival at an optimum cooling rate slightly above 1°C/min when combined with rapid warming. The use of rapid warming and a high (2 M) concentration of glycerol reduces the dependence of survival on cooling rate by broadening the range of rates over which survival is relatively high. (3) Human granulocytes show some sensitivity to dilution stresses since survival depends somewhat on the concentration of glycerol used and the severity of the dilution procedure.

At this time, the reasons for the sharp decrease in cell viability following incubation of frozen-thawed granulocytes at 37°C are not known. One possibility is that the PBS suspending medium used in this study is not suitable for incubation at 37°C. We chose phosphate buffered saline because simple salt solutions are usually as effective as more complex media during freezing, and because they simplify the running of the computer simulations. A second possibility is that some cell injury is simply not expressed at 0°C and remains undetected by the FDA assay until the cells are incubated at 37°C. There is also the possibility that lysosomal enzymes released by a few damaged cells in a sample will cause additional damage in other cells at 37°C, and possibly lead to a chain reaction of injury. Future experiments will hopefully distinguish among these possibilities and aid in solving the vexatious problem of obtaining frozen-thawed granulocytes that can function at 37°C.

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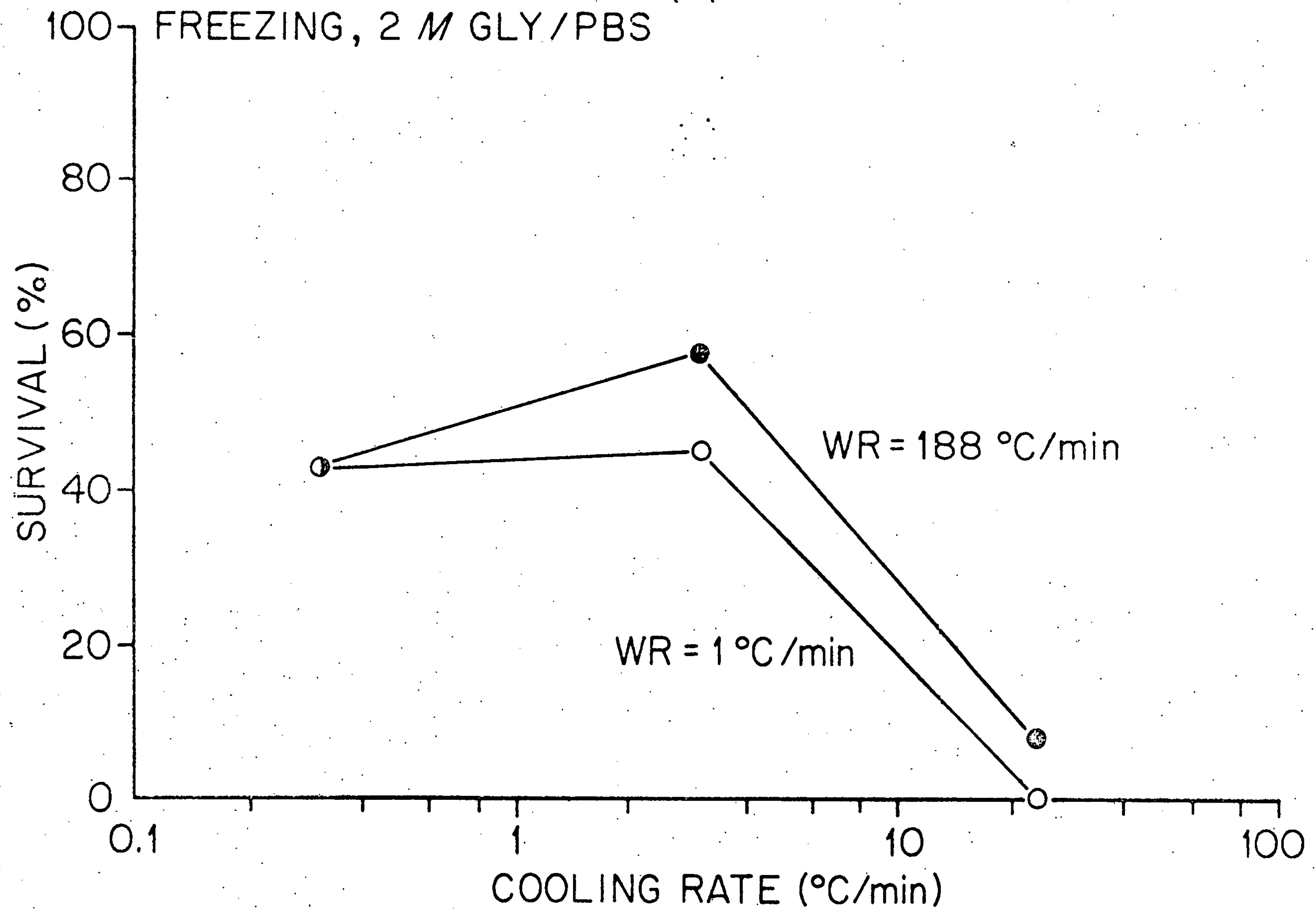
FIGURE LEGEND

Figure 1. Survival of human granulocytes as a function of cooling rate. Cells were suspended in phosphate buffered saline containing 2 M glycerol as a cryoprotectant. After either slow (O) or rapid (●) warming, the cells were diluted free of the additive by a stepwise dilution over a period of 1 hr. Viability was assessed by the FDA assay as described in the text.


FREEZING, 2 M GLY/PBS
SURVIVAL (%)
100
80
60
40
20
0
0.1
1
10
100
COOLING RATE (°C/min)
WR = 188 °C/min
WR = 1 °C/min