MECHANISM OF CRYOPROTECTION BY EXTRACELLULAR POLYMERIC SOLUTES

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ABSTRACT To elucidate the means by which polymer solutions protect cells from freezing injury, we cooled human monocytes to −80°C or below in the presence of various polymers. Differential scanning calorimetric studies showed that those polymers which protect cells best have a limiting glass transition temperature (T'_g) of ~ −20°C; those with a T'_g significantly higher or lower did not protect. Freeze-etch electron micrographs indicated that intracellular ice crystals had formed during this freezing procedure, but remained smaller than ~300 nm in the same proportion of cells as survived rapid thawing. We propose that cryoprotection of slowly frozen monocytes by polymers is a consequence of a T'_g of −20°C in the extracellular solution. In our hypothesis, the initial concentration and viscosity of protective polymer solutions reduce the extent and rate of cell water loss to extracellular ice and limit the injurious osmotic stress, which cells face during freezing at moderate rates to −20°C. Below −20°C, glass formation prevents further osmotic stress by isolating cells from extracellular ice crystals, virtually eliminating cell water loss at lower temperatures. On the other hand, the protective polymer solutions will allow some diffusion of water away from cells at temperatures above T'_g. If conditions are correct, cells will concentrate the cytoplasm sufficiently during the initial cooling to T'_g to avoid lethal intracellular freezing between T'_g and the intracellular T_g, which has been depressed to low temperatures by that concentration. Thus, when polymers are used as cryoprotective agents, cell survival is contingent upon maintenance of osmotic stress within narrow limits.

INTRODUCTION

The cryopreservation of living cells and tissues has become a routine technique in biological and medical laboratories. Cryoprotectants have been divided into two categories; namely, those which penetrate cell membranes and those which do not. The mechanism of cryoprotection by the penetrating agents has been well characterized in the more than thirty years since Lovelock's (1953*a*, *b*) first analysis (cf. Takahashi and Hirsh, 1985). In contrast, the mechanism of cryoprotection by the nonpermeating cryoprotectants, and especially by polymers, has not been similarly resolved despite their effectiveness with a long list of cell types.

Several hypotheses have been proposed. Shortly after their discovery, Nash (1966) noted that they have shared with the penetrating cryoprotectants the property of forming strong and numerous hydrogen bonds. Farrant (1969) suggested that a polymer such as polyvinyl pyrrolidone (PVP) may exhibit enhanced colligative properties at higher concentrations and may protect cells by lowering the external salt concentration at a given subfreezing temperature in a manner similar to low molecular weight cryoprotectants. Pinocytosis of polymers (Persidsky and Richard, 1962) or direct interaction between polymers and cell membranes (Meryman, 1966) have been proposed but subsequent work (Connor and Ashwood Smith, 1973) has argued against these explanations. McGann (1978, 1979) presented a hypothesis he called "squeeze out," in which he postulated that elevated hydroxyethyl starch (HES) concentrations dehydrate cells during freezing and prevent intracellular freezing. Ashwood-Smith et al. (1972), Williams and Harris (1977), and Williams (1983) have suggested that the cryoprotective properties of polymers reside in their ability to alter the physical properties of solutions during the freezing process rather than in direct effects on cell membranes. Körber and Scheiwe (1980), on the basis of thermal analysis, suggested that a certain portion of water is absorbed by HES and kept from freezing. In their hypothesis, the protective action of HES against solute concentration is attributed to its water absorptive capacity and to kinetics, rather than to a postponement of lethal solute concentration to lower temperatures. Some authors have suggested that cell survival is more correlated with the fraction of water unfrozen than with other factors (Mazur et al., 1981). Finally, MacKenzie (personal communication) observed that polymers suppress the formation of salt eutectics whose presence can be lethal.

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Human monocytes can be completely (98 ± 0.8%) cryopreserved by penetrating cryoprotectants (Takahashi and Hirsh, 1985). Certain polymeric cryoprotectants such as HES, PVP, Ficoll, and some dextrans can protect monocytes as well, at a level of 60 to 80% functional recovery. We report finding a critical common thermodynamic property which these polymers share: solutions of each of the cryoprotective polymers which are effective in monocytes undergo a limiting glass transition (T'_g) at ~ -20°C, while polymers which are not cryoprotective have a T'_g above or below this. A limiting glass transition is defined as a glass transition in an aqueous solution which is in equilibrium with ice at the glass transition temperature, having been formed by the removal of H_2O from the solution by the growth of ice crystals alone. Our investigations include the effects of freezing and thawing upon monocytes with and without polymers and upon polymer solutions by themselves, using differential scanning calorimetry (DSC) and freeze-fracture freeze-etch electron microscopy (EM).

METHODS AND MATERIALS

Purification of Human Monocytes

Whole human blood was collected in full units drawn in citrate-phosphate-dextrose anticoagulant by the Washington (DC) Regional Blood Services. Platelet-rich plasma was removed within 2 h of collection by centrifugation at 2,600 *g* for 3 min and the buffy coat, a 40-ml fraction of the top layer of packed cells, collected. The buffy coat was layered on Ficoll-Paque (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ), centrifuged at 400 *g* for 30 min at room temperature and mononuclear cells collected. Monocytes were isolated from this population using a J2-21 centrifuge with a JE-6 rotor (Beckman Instruments, Inc., Palo Alto, CA) and two Sanderson's separation chambers after a procedure modified from Lionetti et al. (1980). The purity of the monocytes was 95% as determined by morphology (May-Grünwald Giemsa) and by nonspecific esterase staining (Technicon Instruments Corp., Tarrytown, NY).

Purified monocytes were suspended in Hanks' balanced salt solution without calcium or magnesium (HBSS; Gibco, Grand Island, NY), containing 5% (vol/vol) fetal calf serum (FCS; M.A. Bioproducts, Walkersville, MD).

Viability Assays

Membrane integrity was checked by the fluorescein diacetate/ethidium bromide assay as described by Martel et al. (1974). In this assay, viable cells show a green fluorescent cytoplasm and dead cells show red fluorescent nuclei under epifluorescent microscopy (Model BHA; Olympus Instruments, New York). Phagocytosis was detected by a modification of the method described by Pantazis and Kniker (1979). In this assay, monocytes are incubated for 30 min with stationary phase wild type *E. coli* which had been opsonized by pooled serum and then stained by acridine orange (Sigma Chemical Co., St. Louis, MO). Monocytes which phagocytized more than five bacteria were regarded as having retained phagocytic function.

The chemotactic response of monocytes was measured by the micropore filter technique. Monocytes suspended in HBSS with Ca^{++} and Mg^{++} ($HBSS^+$) at 2.5×10^6 cells/mL were added to blind well chemotactic chambers (Neuroprobe, Cabin John, MD) fitted with polycarbonate filters containing 5 μm pores as described by Snyderman et al. (1972). As a chemoattractant, the synthetic peptide *N*-formyl-methionyl-leucyl-phenylalanine was used at a concentration of 10 nM. After a 90 min incubation at 37°C in a humidified 5% CO_2 incubator, the filters were removed from the chambers, stained with Diff-Quick (Harleco, Gibbstown, NJ) and the cells counted. Data are presented as mean and standard deviation of the number of monocytes per 1,000× microscope field, 20 fields counted, for triplicate filters.

Freezing and Thawing Methods

Monocytes suspended in HBSS and cryoprotective polymers were distributed into 10 × 75 mm polypropylene test tubes and frozen in a programmed freezer (either Cryo-Med, Mt. Clemens, MI or ULT-80; Neslab Instruments Inc., Portsmouth, NH). Frozen samples were rapidly thawed from either LN_2 or from −80°C by immersion in 37°C water. For slow warming at 1.6°C/min, samples were thawed from −80°C using the program of the ULT-80. To determine the temperature at which injury occurred during slow warming, samples were removed at various temperatures and rapidly warmed in 37°C water. Temperatures were monitored with a fine thermocouple attached to an electronic thermometer (BAT-9; Sensortek, Saddle Brook, NJ).

Freeze-Fracture Freeze-Etch Electron Microscopy

Monocytes suspended in HBSS with or without 20% (wt/wt) HES were placed in a freeze-fracture sample holder, cooled at 2.0°C/min to −80°C and transferred to LN_2. One sample containing HES was transferred to −80°C, warmed at 1.6°C/min to −60°C and plunged again into LN_2. Controls were maintained in LN_2.

Replicas were prepared in a modified Denton DFE-2 freeze-fracture unit as detailed elsewhere (Steere and Erbe, 1983). Specimens were fractured at −150°C and etched at −98°C for 2 min, recooled to −150°C and shadowed. Platimum shadow thickness was monitored electrically and carbon thickness determined visually. Biological material was removed from the replicas with chromic acid (SO-C-88; Fisher Scientific Co., Allied Corp., Pittsburgh, PA). Washed replicas were mounted on electron microscope grids and examined in a JEOL JEM-100 transmission microscope (JEOL USA, Electron Optics Div., Peabody, MA) equipped with a 60° top-entry goniometer stage. Stereo electron micrographs were made with a 10° tilt between pictures in each pair. Since the original electron micrographs have black shadows, they are considered positive. To simulate this, contact internegatives were made on Kodak medium contrast projector slide plates, and these printed.

DSC Measurements

Polymer solutions or cell suspensions were sealed in aluminum pans, weighed, and placed in the scanning calorimeter (DSC-4: Perkin-Elmer Corp., Instrument Div., Norwalk, CT) with computer-aided analysis (TADS). Cooling and warming protocols matched those of the freezing experiments. Seeding of ice formation within the sealed pan was accomplished by equilibrating the sample at a temperature a few degrees below its freezing point, cooling the sample at 10°C/min until an exotherm was seen on the monitor, and then rapidly rewarming it to the equilibration temperature using the "Go To Load" feature. After annealing for at least 5 min, cooling of the sample was resumed according to the protocol.

Chemicals

Hydroxyethyl starch (mol wt 450,000); CV Labs, Riverside, CA. Dextran (mol wt 250,000); J. T. Baker, Philipsburg, NJ. Ficoll (mol wt 70,000); Pharmacia Fine Chemicals. Dextrans (mol wt 10,000–500,000); Pharmacia Fine Chemicals. Dextrans (mol wt. 10,000–500,000); Pharmacia Fine Chemicals. Polyethylene glycol: (mol wt 8,000), J. T. Baker; (mol wt 20,000), Sigma Chem. Co. Polyvinyl pyrrolidone (mol wt 30,000); GAF Chemical Products Div., Lyndon, NJ. Other reagents; Sigma Chemical Co. Human serum albumin; Cutter Biological, Berkeley, CA.

The pH of polymer solutions was adjusted to 7.4 using 2 N NaOH or HCl. HES was dialyzed against H_2O to remove salts, checked on a flame photometer for sodium and potassium and then freeze-dried. PVP was used without dialysis. Its toxicity to monocytes is minimal at pH 7.4.

Other Methods

The relationship between ice crystal size and survivability was analyzed using an exact probability test. All crystals in cells with small ice crystals (≤300 nm) were considered survivable, were counted and scored *s*. On representative cells with large ice crystals which had been presumably killed by them, crystals this small or smaller were scored *s*. Crystals >400 nm were scored *n*. Crystals between 300 and 400 were scored *n* or *s* half of the time. On this basis, we tested the ice crystals seen in Fig. 9, examining the probability that the set seen in Fig. 9 *a* could be drawn from the set of crystals in Fig. 9, *a* and *b*, together plus the probability of drawing all less likely sets of the same size as that of Fig. 9 *a*.

RESULTS

Critical Killing Temperatures of Cells Frozen With and Without HES

Monocytes were suspended in HBSS with or without 20% (wt/wt) HES, equilibrated with ice at −5°C and frozen to various temperatures at 2°C/min. When the temperature had reached a desired value, sample tubes were warmed rapidly in a water bath at 37°C. As Fig. 1 shows, cells not protected by HES lost phagocytic activity progessively as temperature decreased, and activity had disappeared after exposure to −20°C. In contrast, cells suspended in 20% HES still retained 70% of their phagocytic activity after exposure to −20°C, and showed no further loss at −80°C.

Final Freezing Temperature and Its Effect Upon Mortality Due to Slow Warming

Cells were suspended in HBSS containing 20% HES, seeded, cooled at 2.0°C/min to various temperatures and then warmed either at 160°C/min or 1.6°C/min. As shown in Fig. 2, cells warmed rapidly survived under all conditions. If the temperature went below −45°C, cell recovery after slow warming decreased.

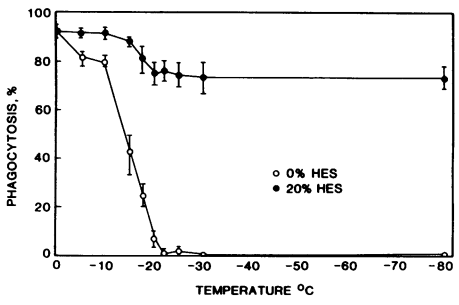

FIGURE 1 Critical killing temperatures of human monocytes frozen with and without HES. Cells were frozen in HBSS solutions with or without 20% (wt/wt) HES, cooled at 2.0°C/min to various temperatures and then warmed rapidly in a 37°C bath. The addition of HES significantly protected cells from freezing injury. Mean + SD (n = 5).

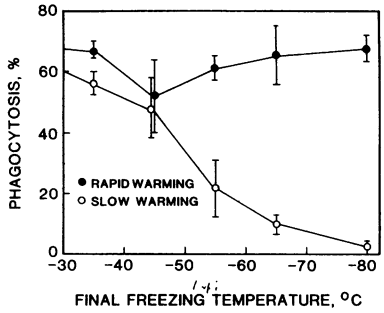

FIGURE 2 The effect of slow warming on mortality. Monocytes suspended in 20% HES were cooled to various temperatures and warmed either at 1.6°C/min or 160°C/min. Cell recovery during slow warming decreased below −45°C.

Effect of Concentration of HES on the Recovery of Phagocytic Activity

Cells were suspended in several concentrations of HES, seeded with ice at −4°C to initiate extracellular freezing, cooled to −80°C at 2.5°C/min, held for 30 min and then warmed at ~200°C/min in a 37°C water bath. As can be seen in Fig. 3, HES did not protect cells from freezing injury at concentrations below 10% (wt/wt). Maximum cryoprotection was obtained above a concentration of 20% (wt/wt). Under these circumstances, ~70% of cells survived.

The Effect of Cooling Rate on Phagocytic Ability

Monocytes suspended in 20% HES solution were seeded with ice at −4°C, cooled to −10°C at 2.5°C/min and then cooled to −80°C at various cooling rates. The samples were held at −80°C for 30 min and then warmed rapidly in a 37°C water bath. Fig. 4 shows a maximum in freezing

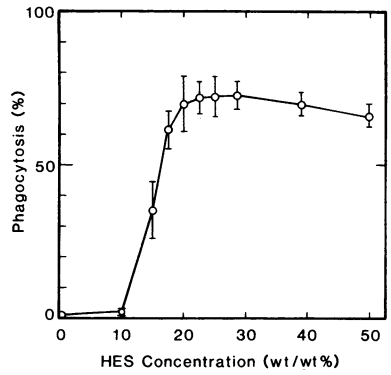

FIGURE 3 Effect of HES concentration on the recovery of phagocytic activity in frozen monocytes. Monocytes were suspended in various concentrations of HES in HBSS. Solutions were seeded at −4°C to initiate extracellular freezing, cooled to −80°C at 2.5°C/min, held for 30 min and then warmed at ~200°C/min in a 37°C water bath. The maximum cryoprotection was obtained above a concentration of 20% (wt/wt).

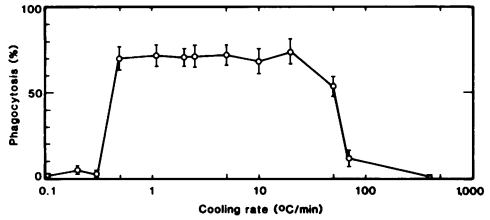

FIGURE 4 The effect of cooling rate on phagocytic activity. Cells were suspended in HBSS solution with 20% HES, seeded at −4°C, and cooled to −60°C at various rates. The samples were held for 30 min at −80°C and than warmed rapidly in a 37°C water bath. Maximum cryoprotection was obtained at cooling rates between 1 and 20°C/min.

survival when the cooling rate was between 0.5°C/min and 20°C/min.

The Effect of Quenching Temperature Upon Phagocytic Activity

Cells suspended in HBSS containing 20% HES were equilibrated with ice, frozen at 2.5°C/min to various temperatures and then either warmed rapidly or plunged into LN_2 (quench cooling). After 30 min storage, they were thawed in a 37°C water bath. Fig. 5 shows that survival improved if the quenching had begun at temperatures below −20°C after slow freezing to that temperature. Below −30°C, there was no difference between cells that were warmed directly or warmed after quenching in LN_2.

The Critical Temperature for Slow Warming

Cells suspended in 20% HES solution were frozen to −80°C at 2.5°C/min, plunged into LN_2, and stored for 30 min. Samples were then transferred to the −80°C bath, in which they warmed at 1.6°C/min. At intervals, samples were removed and warmed rapidly by plunging them into a 37°C water bath. As Fig. 6 shows, the viability decreased sharply if the temperature to which the samples had been slowly warmed exceeded −70°C.

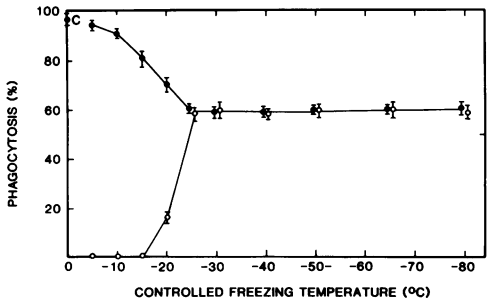

FIGURE 5 The effect of quenching temperature upon phagocytic activity. Cells suspended in 20% HES were cooled at 2.5°C/min. At a certain temperature during cooling, the cells were either warmed rapidly (●) or plunged into liquid nitrogen and then warmed rapidly in a 37°C water bath (0). Data are mean ±1 SD ($n = 3$). The survival of cells after plunging into liquid nitrogen was maximal after the slow cooling had proceeded to −20°C, at which the results were indistinguishable from cells that had been warmed directly.

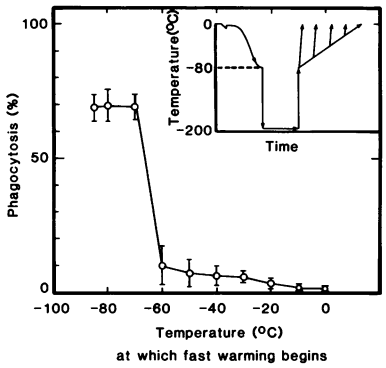

FIGURE 6 The critical temperature for slow warming. Monocytes suspended in HBSS containing 20% HES were cooled at 2.5°C/min to −80°C and plunged into liquid nitrogen. They were then placed in an −80°C bath and warmed at 1.6°C/min. Subsequently, cells were removed at various temperatures and warmed rapidly in a 37°C water bath. Phagocytic ability was lost if cells had been warmed slowly above −70°C.

The Thermal Behavior of Polymer Solutions and Its Relationship to the Cryoprotective Effectiveness of Various Polymers

A 20% (wt/wt) solution of HES in HBSS was cooled in the calorimeter to −160°C at 2°C/min and rewarmed at the same rate. This record is presented in Fig. 7, *inset*. On warming, the limiting glass transition of the solution is observed at ~ −20°C; the equilibrium melting of ice appears above this temperature.

Monocytes were suspended in solutions of various poly-

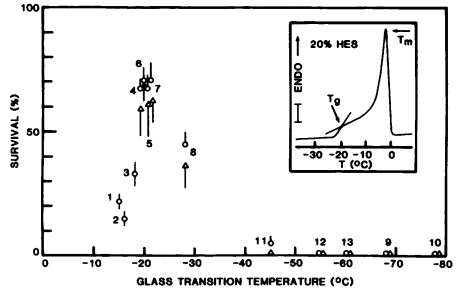

FIGURE 7 Cryoprotection of monocytes by various extracellular agents. Monocytes were frozen at 2.5°C/min to −80°C in 20% solutions of various nonpenetrating solutes, held for 18 h and then warmed rapidly in a water bath at 37°C. Survival (mean ± 1 SD) is plotted as a function of the temperature of the glass transition (see inset). Note that the maximum survival was observed when cells were suspended in agents which have a glass transition at ~ −20°C. (*Inset*) The DSC thermogram for 20% Hydroxyethyl starch in Hanks balanced salt solution. The limiting glass transition was obtained by cooling the sample at 2.5°C/min, and recording the thermogram while warming the sample at the same rate. Key. 1: dextran, 250 kD. 2: dextran, 500 kD. 3: dextran, 450 kD. 4: dextran, 40 kD. 5: dextran, 10 kD. 6: hydroxyethyl starch, 450 kD. 7: Ficoll, 70 kD. 8: polyvinyl pyrrolidone, 30 kD. 9: polyethylene glycol, 8 kD. 10: polyethylene glycol, 20 kD. 11: sucrose. 12: glucose. 13: sorbitol. The symbol indicates assay by chemotaxis; the 0 symbol by phagocytosis.

TABLE I
REPEATING OF FREEZING: EFFECT OF REPEATED FREEZING TO −30°C ON THE RECOVERY OF PHAGOCYTIC ABILITY

Cryoprotectant concentration	First freezing	Second freezing	Third freezing
HES 20% *(wt/wt)*	63.0 ± 5.7*	26.5 ± 9.8	0.0 ± 0.0
Me_2SO 7.5% *(vol/vol)*	93.7 ± 3.2	90.8 ± 3.4	88.8 ± 1.8

Cells were suspended in HES solution (20%) or Me_2SO (7.5%), cooled to −30° after seeded at ≥−6°C, then warmed rapidly in a 37° water bath. After the ice melted, cells were frozen again under the same conditions. This procedure was repeated three times. Control cells (no freezing): 95.3 ± 0.6% phagocytosis.
*Mean ± SD (n = 3).

mers, seeded with ice at −5°C, cooled to −80°C at 2.5°C/min in a programmed freezer, plunged into LN_2, stored for 18 h, and warmed rapidly in a 37°C water bath. After cells were washed twice in HBSS, phagocytic activity was determined. In addition, thermograms were made of the suspending solutions without cells. All these data are summarized and correlated in Fig. 7. Polyethylene glycol (9 in Fig. 7) could not protect monocytes. Its limiting glass transition is at ~ −70°C, conspicuously below that of the cryoprotective polymer solutions, whose limiting glasses melt at ~ −20°C. Among dextrans of various molecular weights, the higher molecular weight materials with glass transitions well above −20°C (1, 2, 3 in Fig. 7) were also less effective. The monomeric compounds (11, 12, 13 in Fig. 7), with transition temperatures lower than −50°C, were ineffective as cryoprotectants.

Attempts were made to correlate injury to the unfrozen fraction of water in various cryoprotective solutions. Integrating the melting peaks of the DSC thermograms of the test solutions produced evidence for a negative correlation: the less water freezing out, the worse the cryoprotection. Representative analyses from our data are: 56.4% total water loss to ice in 20% sucrose, (T'_g = −45) 64.2% in 20% Dextran 500,000 mW (T'_g = −15) and 67.0% in 20% HES (T'_g = −20). Survival was near zero in sucrose (11 in Fig. 7), low in dextran (2 in Fig. 7), and maximal in HES (6 in Fig. 7).

TABLE II
ADDITIVE CRYOPROTECTION BY THE COMBINATION OF HES AND Me_2SO

Concentration (7)		Phagocytosis
Me_2SO	HES	%
0	0	0.0 ± 0.0*
1	0	2.5 ± 1.7
2	0	23.5 ± 6.4
3	0	71.7 ± 11.6
4	0	88.5 ± 2.9
5	0	93.5 ± 2.2
7	0	93.8 ± 2.8
0	20	70.8 ± 2.2
1	20	86.5 ± 1.3
2	20	91.3 ± 4.3
3	20	94.3 ± 1.4
4	20	93.3 ± 1.7
5	20	94.0 ± 2.2
7	20	94.0 ± 1.8

Cells were suspended in each solution, seeded at ≥−6°C, cooled to −80°C at 2.5°C/min, and held for 1 h. Then samples were thawed rapidly in a water bath at 37°C. Me_2SO was removed from cells slowly by the addition of washing solution (HBSS containing 5% FCS). Control's (no treatment) phagocytic ability was 94.5 ± 2.1%.
*Mean ± SD (n = 4).

Effect of Multiple Freezes on Monocytes in HES or DMSO Solutions

Cells were suspended in solutions containing either 7.5% DMSO or 20% HES, equilibrated with ice at −5°C, cooled at 2.5°C/min to −30°C, held at that temperature for 10 min and rapidly warmed in a 37°C water bath. This cycle was repeated thrice. As Table I shows, cells in HES had lost virtually all phagocytic activity after the third cycle, while cells suspended in DMSO solutions showed no significant loss of function.

Cryoprotective Effect of Combinations of HES and DMSO

Cryoprotected cells were equilibrated with extracellular ice at above −6°C, cooled to −80°C at 2.0°C/min, held at that temperature for 1 h and then thawed rapidly in a 37°C water bath. Table II summarizes the effects of combining

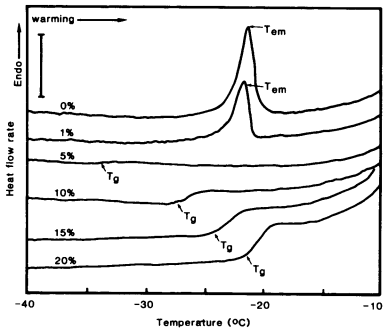

FIGURE 8 The suppression of eutectic crystallization by HES. Various concentrations of HES were dissolved in HBSS and cooled at 2.5°C/min in a DSC. These records of the thermograms produced by warming at the same rate indicate that the NaCl eutectic melting at −21.1°C is suppressed by ≥5% HES. Instead, the solution vitrified at progressively higher temperatures as a function of increasing HES concentration.

these two cryoprotectants. When DMSO was used alone, increased concentration gave increased cryoprotection up to 5%. When 20% HES was used in conjunction with DMSO, maximal cryoprotection was obtained when the DMSO concentration was at or above 2%.

Suppression of the NaCl Eutectic by HES

In low concentrations of HES, the calorimeter showed, among other things, the eutectic melting of NaCl (Fig. 8). The presence of HES suppressed this thermal event, and above 5% HES suppressed it completely. Instead, a glass was formed whose transition temperature increased with increasing concentration of HES.

Freeze-Fracture Freeze-Etch Electron Microscopy

Cells suspended in 20% HES solutions were frozen extracellularly in an aluminum holder, cooled to −80°C at

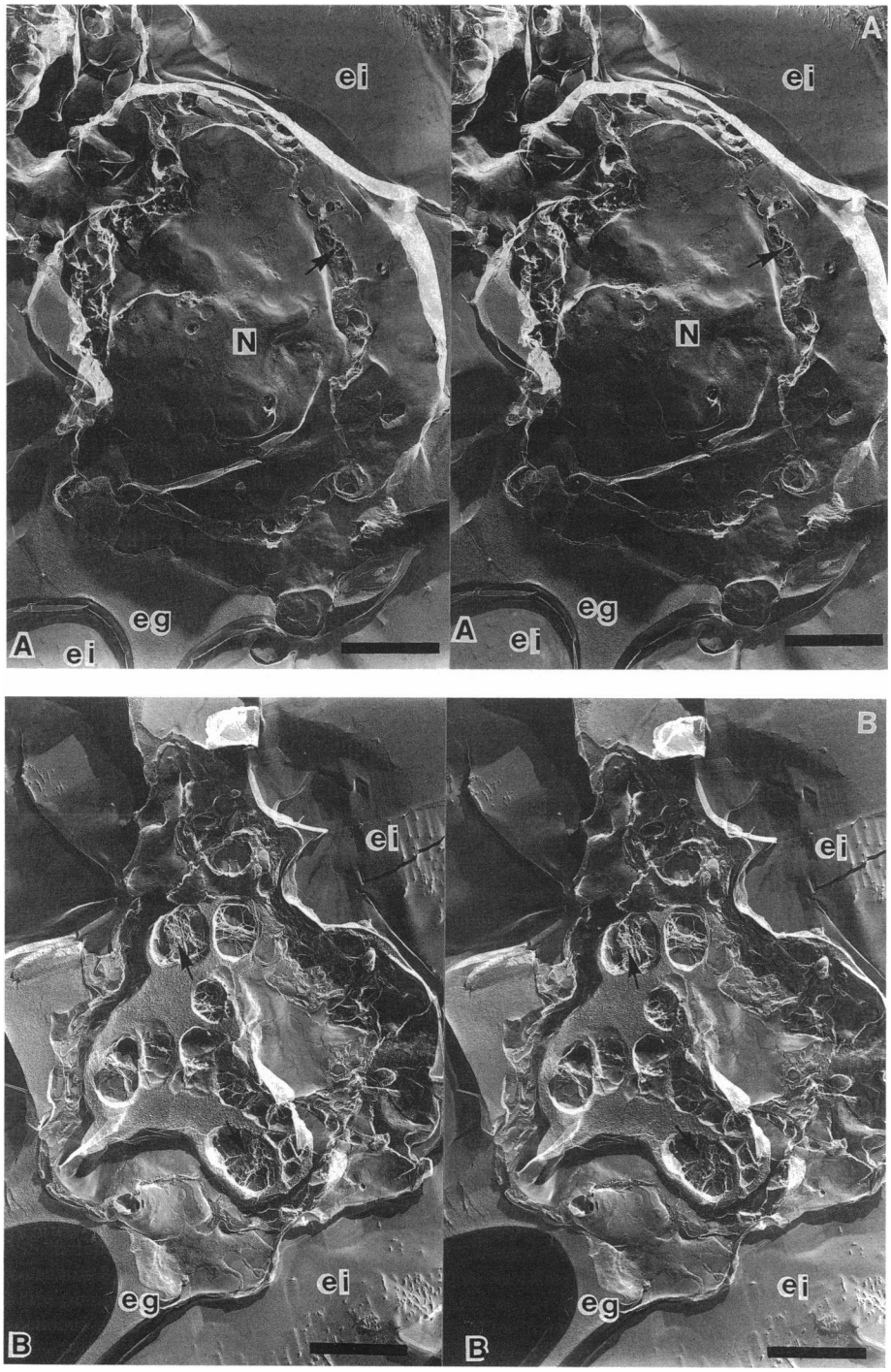

FIGURE 9 Freeze-etch electron microscopy of cells frozen in HES solution (*A*) Cells suspended in 20% HES solution were frozen to −80°C at 2.5°C/min and plunged into liquid nitrogen. (*B*) Same as *A*. Observations of replicas indicated that ~30% of cells (at least 200 cells were counted) had large ice crystals as shown in *B* and ~70% of cells had small ice crystals as shown in *A*. (*C*) Cells were frozen to −80°C at 2.5°C/min, plunged into liquid nitrogen, warmed rapidly to −80°C, warmed to −60°C at 1.6°C/min, and held for 5 min at −60°C, and then plunged into liquid nitrogen. Most of the cells (>90%) now contained large ice crystals. (*D*) Monocytes in Hanks' balanced salt solution without HES were frozen to −80°C at 2.5°C/min, plunged into liquid nitrogen. Bars indicate 1 μm. Magnification: 13,600. *N*: nucleus, *eg*: extracellular glass, *ei*: extracellular ice. Arrows indicate ice crystals formed intracellularly. About 70% of the cells examined displayed the small intracellular ice crystal structure correlating well with the number of cells surviving this treatment after fast warming. When cells are warmed slowly from −80° to −60°C, virtually all cells then show massive intracellular ice formation and survival approaches zero. Thus, it appears that the partially dehydrated intracellular solutions form unstable glasses and a small amount of ice when cooled to −80°C. Warming to −60°C melts the glass and allows ice formation to proceed. Presumably, when warming from −80°C is rapid, insufficient time for ice growth is available. When cells were frozen at 2.5°C/min to −80°C in HBSS alone, mortality was 100% but no intracellular ice formed.

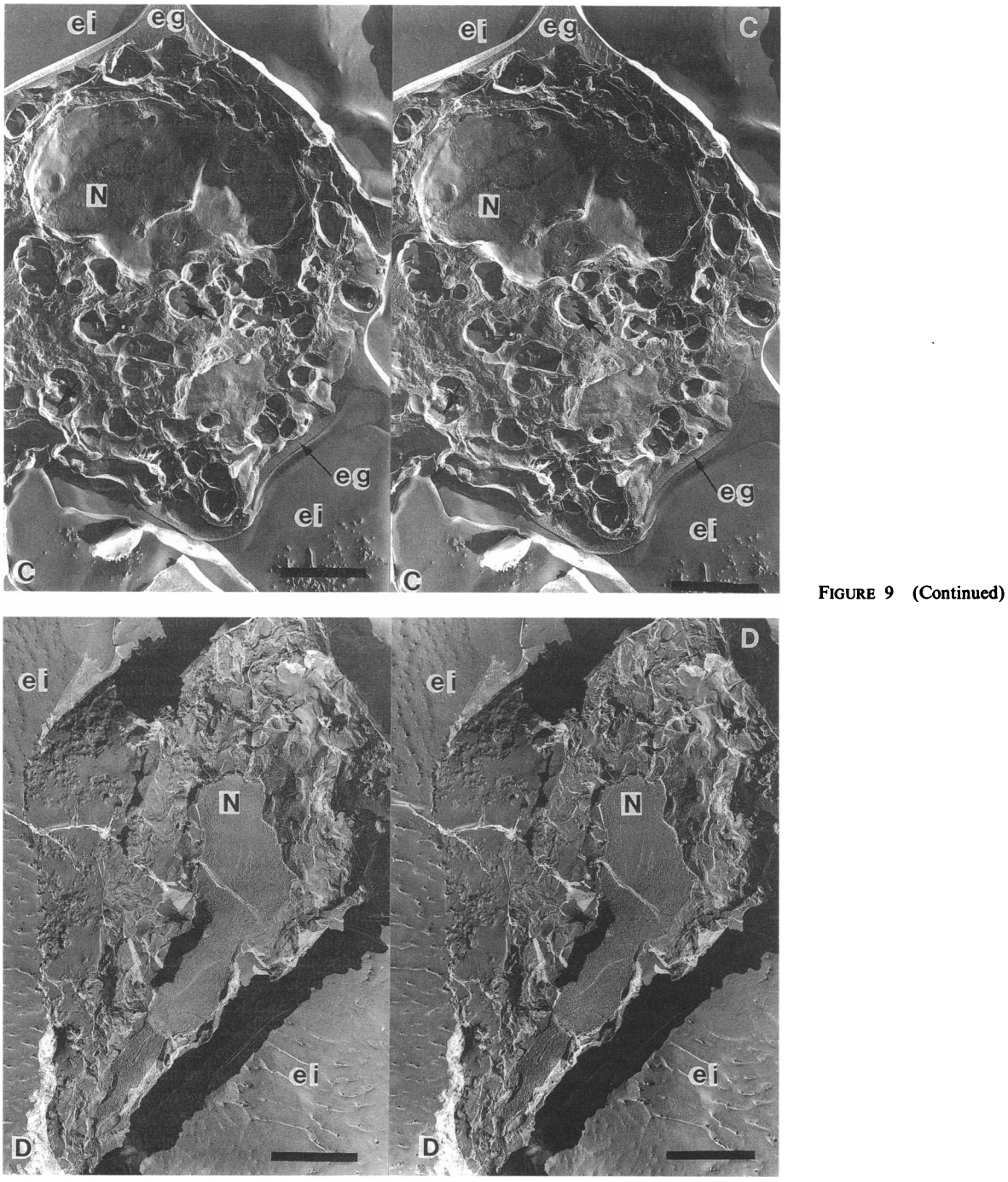

FIGURE 9 (Continued)

2.0°C/min and plunged into LN_2. One sample remained as a control, while the other was warmed at 1.6°C/min to −50°C, held for 5 min and again plunged into LN_2. These samples were freeze-fractured and etched at −98°C for 2 min and replicated. As shown in Fig. 9 *b*, ~30% of the cells frozen slowly in HES solutions but not rewarmed showed large cytoplasmic ice crystals (diameter = 432 ± 250 nm; $n = 13$). In the other 70% of cells (Fig. 9 *a*), crystals had formed but were small (diameter 165 ± 93 nm; $n = 13$). All cells in the sample warmed slowly to −60°C showed large cytoplasmic ice crystals (diameter 391 ± 150 nm; $n = 35$), as demonstrated by Fig. 9 *c*. The distribution of injurious and noninjurious ice crystals was examined using an exact probability test. The probability that the set of ice crystals seen in Fig. 9 *a* could be drawn from the crystal set in Fig. 9, *a* and *b* plus the probability of drawing all less likely sets of the same size as that of Fig. 9 *a* is ≤0.006. The equivalent probability when comparing Fig. 9, *a* and *c* is ≤0.005. In the comparison between Fig. 9, *b* and *c*, two cells that were most likely to have been killed, the probability is >0.5.

Fig. 9 *d* shows cells that were frozen at 1.6°C/min to

−80°C in HBSS with no cryoprotection at all. All cells died upon warming after treatment, but none showed evidence of intracellular ice.

DISCUSSION

Compared with human granulocytes (Takahashi et al., 1985), monocytes are relatively resistant to the osmotic stress that cells face during freezing. At 0°C they can tolerate 1.4 osm for 10 min, roughly equivalent to the resistance in human red cells. This is 4.6 times their isosmotic concentration, with a freezing point depression of 2.6°C. In the absence of cryoprotectant monocytes are progressively injured when frozen, losing all function after freezing to −20°C (Fig. 1).

Freezing rates between ~0.5°C/min and 50°C/min are tolerated in suspensions fully cryoprotected with HES (Fig. 4). If the medium contained ≥20% HES (Fig. 3), 70% of cells remaining functional after freezing to −20°C remained functional after continued slow cooling to −80°C, so long as warming was rapid (Fig. 2). Additionally, cell survival remained at 60% after a plunge directly into liquid nitrogen, so long as the sample had first been slowly frozen to some temperature below ~ −25°C (Fig. 5).

When cells cryopreserved with polymers alone were warmed at 1.6°C/min from −80°C, they lost phagocytic activity (Fig. 6). This injury did not appear during rapid warming, suggesting that cells cooled to below −80°C under optimal conditions contained intracellular ice crystals, but that these were small and did not grow significantly during rapid warming. Many types of cells have been shown to survive small intracellular ice crystals (e.g., Asahina, 1965). Slow warming above −80°C would allow these small crystals to grow by recrystallization, and to become lethal. The size difference between inocuous and injurious ice crystals suggests mechanical destruction of the cytoskeleton as a mechanism of freezing injury.

Because of the viscosity and density of polymer solutions, we were not able to obtain suspensions of cryopreserved monocytes containing enough cells to verify in the calorimeter the formation of intracellular ice at low temperatures. Instead, we have used the electron microscope to confirm the growth of intracellular ice. (Fig. 9 *a*). The electron microscopy showed that even in cell preparations frozen under optimal conditions, 30% of cells have intracellular ice crystals larger than 400 nm in diameter, whereas slow warming produced ice crystals this size in more than 95% of cells (Fig. 9). These data correlate well with our cell survival studies, wherein 30% of cells were injured in 20% HES even after fast warming. Cell mortality doubled if cells were frozen in HES solutions repeatedly, from 30 to 70% during the second freezing. Virtually all cells were killed by the third freezing (Table I). In contrast, repeated freezing in DMSO solutions produced little additional damage. Thus, the polymers protect differently from the penetrating cryoprotectants.

DSC records of the 20% HES solution show that there is a limiting glass transition at ~ −20°C (Fig. 7, *insert*). This temperature is quite high when compared with penetrating cryoprotectants such as DMSO or glycerol which have limiting T'_gs below −83°C. The other polymers that protect monocytes from freezing injury such as dextran, PVP, and a Ficoll, also have T'_gs near −20°C. On the other hand, polyethylene glycol, which is used as a cryoprotectant for some cell types, has a glass transition at −70°C. Significantly, this polymer cannot protect monocytes. Nonpolymeric chemicals such as sucrose, which penetrate cells too slowly to be effective intracellularly but which are nevertheless sometimes used as cryoprotectants, also display a T'_g below −50°C. They were ineffective in preserving human monocytes.

Integrating the melting curves gives accurate values of the ice formed in each cell preparation, and these data allowed us to demonstrate that in human monocytes, injury does not occur when a critical fraction of water is removed to ice (cf. Mazur et al., 1981). In fact, we found a negative correlation, the less water freezing out, the worse the cryoprotection.

Our evidence indicates that a T'_g in polymers at ~ −20°C is essential to their ability to cryoprotect, independent of polymer composition. We can speculate how this attribute affects the physical condition of extracellular and intracellular solutions during freezing. When ice forms in the extracellular space it concentrates the extracellular solution. This in turn extracts water from cells, concentrating cellular contents and subjecting the cells to an osmotic stress. It also increases extracellular viscosity, reducing the rate at which water can be withdrawn from cells to ice crystals located some micrometers away.

The effect of freezing upon the viscosity of solutions near T'_g is surprising. We do not have data on HES, but an abundance of data on other starch hydrolysates has been published by Slade and Levine (1987). The viscosity in the solid glass may be 10^{12} Pa.s, but viscosity decreases approximately tenfold for each three degrees increase in temperature. Thus, cells will be more or less in osmotic equilibrium with the extracellular solutions until they are near to the T'_g, when they will rather suddenly begin to supercool significantly. This can also be inferred from the EM data, as cells frozen in HES show supercooling, evidenced by intracellular ice, while unprotected frozen cells do not (Fig. 9). Viscosity changes would also explain the constraints upon cooling rate (Fig. 4): if the rate is too high, cells would become overly supercooled, and if it is too low, there would be sufficient time for excessive osmotic stress to develop. As the temperature decreases and the concentration increases, the extracellular glass transition temperature rises; eventually at a polymer concentration of ~70% (wt/wt), no more water can be frozen out because the

liquidus curve closely approaches the glass transition temperature. It is this intersection which has been called the limiting glass transition, T'_g, and its temperature is highly dependent upon the solution composition.

While the T'_g may be insensitive to the initial polymer concentration, cell osmotic injury is not. Because of the high molecular weight of the cryoprotective polymers, cells exposed to an isomotic solution containing 20% of a polymer experience only a small increment of osmotic stress. According to our hypothesis, even freezing this 20% solution to its T'_g at 70% concentration should not exceed limits of short-term osmotic tolerance, which in these cells is ~4.6 times isosmotic. These limits would, however, be reached or exceeded during the freezing of a solution whose initial concentration was ≤15%. Thus, the effect of polymer concentration on survival seen in Fig. 3 can be explained. The presence of what would normally be considered suboptimal amounts of DMSO appears to ameliorate the remaining osmotic stress (Table II).

While the viscous restriction of flow of intracellular water to extracellular ice may be an important effect, some osmotic shrinkage appears to be essential to cryopreserving monocytes, as solutes with a T'_g above −20°C decrease survival below the optimum (Fig. 7) and rapid freezing from too high a temperature is injurious (Fig. 5). To understand this, consider the effect of the extracellular events upon the physical properties of the protoplasm. It has been demonstrated that cells often lack efficient intracellular ice nuclei and that in the absence of extracellular ice, cells will supercool nearly to the theoretical limit for homogeneous nucleation, ~ −40°C (Burke et al., 1976). It has also been shown that solutes which depress the freezing point also depress the homogeneous nucleation temperature, but by about twice as much (Rasmussen and MacKenzie, 1971). Thus, in a cell lacking heterogeneous nuclei whose cytoplasm has been concentrated until its freezing point is ~ −15°C, the temperature at which intracellular ice will spontaneously form will have been depressed to at least −70°C. This is close to the T'_g of the glasses measured in living cells and in model protein solutions, so that any nuclei formed during cooling would not have the opportunity to grow until the temperature had been subsequently raised above −70°C (Fig. 6). By the analogous argument, too little concentration of cytoplasm during the initial stages of freezing will result in significant intracellular ice formation during cooling to −70°C, and this development of intracellular ice can explain the failure to protect by polymer solutions whose glass transition is much above −20°C.

Because of the high viscosity and density of the polymer solutions, we could not concentrate cells to the extent that the changes in intracellular ice content and structure were detectable by DSC. However, we were able to observe in the electron micrographs (Fig. 9) that 70% of the cells contained ice crystals smaller than 300 nm. This would indicate that in the cytoplasm of cells cooled at 2°C/min to −80°C or below, ice crystal growth can be suppressed, but not ice nucleation. Fig. 2 implies that intracellular ice nuclei may start to form at temperatures as high as −45°C. In cells containing ice nuclei formed at some low temperature and subsequently warmed slowly, ice crystal growth will harm them, which may explain why all known cell types cryopreserved by polymers require relatively rapid cooling and high warming rates (Doebbler et al., 1966).

The multiple freezing experiment (Table I) suggests that a possible explanation for the loss of 30% of cryoprotected cells even under optimal conditions is the topographical distribution of cells in the polymer solution rather than the selective elimination of a particularly sensitive subpopulation. The injured 30% could be surrounded by a layer of HES solution thinner than average, bringing them too close to ice crystals, reducing the diffusion path below a critical value and exposing them to an excessive osmotic stress. Alternatively, 30% of the cells could have frozen intracellularly because of an unusually thick layer of HES solution between them and the nearest neighbor ice crystal; these would be more supercooled than average and homogeneous (or heterogeneous) intracellular nucleation would occur at a relatively high temperature, producing much ice which could grow into large crystals. The upper limits placed on osmotic stress by the presence of 20% polymer argue against the former. The absence of ice in freeze-etch electron micrographs of unprotected cells frozen to −80°C (Fig. 9 *d*) supports the alternative that the cells killed in 20% HES during cooling are overly supercooled.

Regarding the possibility that polymers protect by suppressing the NaCl eutectic, note (Fig. 8) that one needs polymer concentrations far in excess of those that suppress the eutectic to obtain significant cryoprotection.

CONCLUSION

Monocytes, which are fully cryoprotected by the presence of 7% DMSO, can be cryoprotected to ~70% survival by extracellular polymeric solutes alone. Survival is maximum when the T'_g of the polymeric extracellular solution is ~ −20°C, but drops off sharply at both higher and lower values. Polymers also potentiate the effect of the penetrating cryoprotectants such as DMSO. The percentage of water that crystallizes out of the sample correlates positively with survival.

We propose that cryoprotective polymer solutions protect monocytes by the following mechanism: (*a*) because their viscosity becomes very great as their temperature approaches T'_g, polymers allow cells to supercool a moderate amount between 0°C and the T'_g of the extracellular solution, provided that the initial polymer concentration is sufficient to impose a tolerated upper limit on water loss. Further injurious water loss from the cells at temperatures below T'_g is eliminated by the solidification of the solution

between the cells and extracellular ice, maintaining osmotic stress at a tolerable level; and (*b*) at temperatures below −70°C, high intracellular viscosities suppress the growth of intracellular ice crystals, crystals which can grow if the temperature is raised slowly to −60°C or above.

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