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Antifreeze protein modulates cell survival during cryopreservation: Mediation through influence on ice crystal growth

JOHN F. CARPENTER AND THOMAS N. HANSEN

CryoLife, Inc., 2211 New Market Parkway, Suite 142, Marietta, GA 30067

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ABSTRACT **Antifreeze proteins (AFPs) are extremely efficient at inhibiting ice recrystallization in frozen solutions. Knight and Duman [Knight, C. A. & Duman, J. G. (1986) *Cryobiology* 23, 256–263] have proposed that this may be an important function of the proteins in freeze-tolerant organisms. We have tested this proposal *in vitro* by characterizing the influence of AFP on the recovery of cryopreserved cells, which often can survive cooling and yet subsequently be damaged by ice crystal growth during warming. Relatively low concentrations (e.g., 5–150 μg/ml) of winter flounder (*Pseudopleuronectes americanus*) AFP enhance survival of red blood cells cryopreserved in hydroxyethyl starch solutions. This effect is most apparent in samples warmed at suboptimal rates, i.e., where ice recrystallization would be exaggerated. Cryomicroscopy demonstrates that AFP inhibits ice recrystallization in the extracellular regions during the latter stages of the warming cycle. AFP concentrations that enhance survival of red cells confer partial inhibition of recrystallization. Relatively high concentrations of AFP (e.g., 1.54 mg/ml) are much more effective at inhibiting extracellular recrystallization. However, extensive growth of ice around the cell, and concomitant cell damage, is noted. The mechanism for this AFP-induced ice growth is unknown. We propose that there is a delicate balance between AFP-induced enhancement of cell preservation and AFP-induced enhancement of cell damage and that this balance hinges on the degrees of inhibition of ice recrystallization and of preferential growth of ice around the cells. We conclude that, under appropriate conditions, one of the proposed functions of AFPs in nature can be emulated, and perhaps have application, in cryopreservation of materials of biomedical interest.**

Research to date has suggested two distinctly different major functions for antifreeze proteins (AFPs) in nature. First, in certain teleost fish from polar and north temperate regions and in some overwintering insects, the proteins noncolligatively lower the freezing point of the body fluids by at least 1°C, without significantly affecting the melting point (1–7). The proteins appear to function solely as antifreezes, since exposure to temperatures that lead to ice formation results in the death of the organism (3, 4, 7, 8).

AFPs are also produced by some species of insects, centipedes, intertidal molluscs, and plants that can survive freeze–thawing (4, 7, 9–11). AFPs isolated from both fish and insects, even at concentrations of <1 μg/ml, are extremely effective at inhibiting recrystallization of ice (11–13). Recrystallization can occur as frozen samples are warmed to temperatures at which there is sufficient kinetic energy for the migration of water molecules from one ice crystal to another and is, thus, very rapid near the melting point (11). As explained by Knight and Duman (11), this process occurs, in part, because the overall interfacial surface area and free energy of the system are reduced when some ice crystals grow, as others shrink and disappear. Those researchers have suggested that the capacity of AFP to inhibit recrystallization might aid in protecting freeze-tolerant organisms during long-term exposure to subfreezing temperatures and recurrent bouts of freeze–thawing in winter (11).

The goal of the present study was to use a cell model to test this suggestion *in vitro* and to discern how such a lesson from nature might be used to advantage in biomedical cryopreservation. Damage to cryopreserved cells has often been noted when samples are warmed at suboptimal rates during the thawing cycle, an effect that is ascribed to ice recrystallization (14, 15). The model system we chose for these studies consisted of human red blood cells, which were rapidly cooled (*ca.* 600°C/min) in a solution of an extracellular cryoprotectant, hydroxyethyl starch (HES). This system is well suited for testing the influence of AFP on cell survival because the extremely small ice crystals formed should readily recrystallize during warming. Indirect evidence for such damage comes from the observation that red blood cells that are cryopreserved in HES must be thawed very rapidly to optimize cell survival (16). Our hypothesis was that recrystallization and the concomitant cell damage should be attenuated in the presence of AFP.

We found that relatively low concentrations of AFP (5–160 μg/ml) greatly enhanced the survival of red cells that were cryopreserved in HES and thawed by warming at a suboptimal rate. Surprisingly, however, we found that AFP at a high concentration (1.54 mg/ml) induced additional damage to the red cells and that this effect was due to preferential growth of ice around the cells during warming.

MATERIALS AND METHODS

Materials. Purified (>99% homogeneity) AFP was a gift from J. E. Villafranca (Agouron Pharmaceuticals, La Jolla, CA). The recombinant protein was produced by expression of a synthetic gene in *Escherichia coli*. The protein has a molecular mass of 3300 Da, consists of 37 amino acids, and has an amino acid sequence identical to that of the natural, type I AFP from winter flounder (*Pseudopleuronectes americanus*), which is designated HPLC-6 (see ref. 6).

HES (average M_r 450,000; 7 ethyl substitutions per 10 anhydroglucose residues; Kendall McGaw Laboratories, Santa Ana, CA) was used as received and was prepared as a 30% (wt/vol) solution in distilled water.

Preparation and Cryopreservation of Red Blood Cells. Human blood was collected from healthy volunteers into heparinized tubes and centrifuged (2000 × *g*, 10 min). The buffy coat was removed and the remaining red cells were recentrifuged. Sufficient plasma was removed from the supernatant to give a final hematocrit of ≈70%. To an aliquot of the red cell suspension was added an equal volume of 30% (wt/vol) HES solution containing various concentrations of AFP. The values given for the final AFP concentrations are based on the assumption that the protein and HES are diluted 1.3-fold when mixed with the extracellular solution present in

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Abbreviations: AFP, antifreeze protein; HES, hydroxyethyl starch.

a preparation of red blood cells at 70% hematocrit. Also, since the HES contains NaCl, the final extracellular solution in the red cell suspension was approximately isosmotic with the red cell cytosol (i.e., 290 mosmol/kg of water). Aliquots (0.4 ml) of the suspension were placed into 1.5-ml Eppendorf test tubes. The tubes were immersed into liquid nitrogen and stored overnight in liquid nitrogen vapor. To assess the influence of warming rates, samples were warmed extremely rapidly by immersion into a 45°C water bath or extremely slowly in air at room temperature. In one experiment, intermediate warming rates were tested by immersing sample tubes in water baths set at 23 and 37°C. After thawing, the degree of hemolysis was measured (17) either immediately or after a 1:40 dilution of the sample with phosphate-buffered saline (137 mM NaCl/2.7 mM KCl/1.5 mM KH_2PO_4/8.1 mM Na_2HPO_4, pH 7.5 at 25°C). Each value given is the mean ± SD for triplicate samples of cryopreserved red blood cells.

Measurement of Cooling and Warming Rates. The tip of a fine, needle-shaped thermocouple was placed approximately into the geometric center of the 0.4-ml aliquot of HES/red cell suspension. The temperature changes during cooling and subsequent rewarming were monitored by a digital thermometer and a chart recorder. The cooling rate was determined from the linear section of the trace (i.e., between −20 and −120°C) obtained for samples immersed into liquid nitrogen. The measured rate was 606 ± 15°C/min (mean ± SD, $n = 4$). For determination of warming rates, each trace was arbitrarily subdivided into sections in which the slopes were essentially linear. For samples thawed in air at room temperature the warming rate was approximately 40°C/min between −130 and −50°C, 25°C/min between −50 and −10°C, and 2.5°C/min for the remainder of the thawing cycle. For samples thawed in a 45°C water bath the warming rate was approximately 450°C/min between −130 and −10°C, and 30°C/min during the remainder of the thawing cycle. These values were used in the cryomicroscopy experiments described below.

Cryomicroscopy. In these experiments, mimicking the test-tube studies described above, alterations in the ice crystal size were monitored visually with a light microscope (Olympus BH-2) equipped with polarized optics. HES/red cell mixtures were prepared as described above. The final concentrations of AFP tested were 0, 62 μg/ml, and 1.54 mg/ml. The mixture was centrifuged (2000 × g, 10 min), and a 1.5-μl aliquot of the supernatant and some of the cells in the pellet (i.e., such that about 10–50 cells were in the microscope's field of view) were placed onto a cryostage (Interface Techniques, Cambridge, MA) and covered with a glass coverslip. This reduction in cell density was necessary for observation of changes in the extracellular region. Under the conditions used, altering the hematocrit would not be expected to influence cell survival during cryopreservation (18).

The computer interface and software for the cryostage allow control of warming and cooling rates. All samples were cooled at 600°C/min to −133°C. In one set of experiments, the warming rates simulated those determined for test-tube samples that were thawed in air at room temperature. Micrographs were prepared with a Sony video camera (model CCD-IRIS) coupled to a Sony video printer (model UP-5000). Images were collected of samples at −133°C, immediately prior to the initiation of warming, during the warming cycle at −11 and −4°C, and at the melt. In another set of experiments, the warming rate simulated that for test-tube samples thawed by immersion in a 45°C water bath. Micrographs were taken at about −1°C during the warming cycle, just prior to the start of the melt.

RESULTS

Initially, we compared conditions that provided the greatest opportunity for ice recrystallization damage to the cells (i.e., slow warming) with those that should minimize this stress (i.e., rapid warming). Thus, any protection conferred by AFP-induced inhibition of recrystallization would be obvious. Hemolysis of red cells thawed by rapid warming in a 45°C water bath was only about 3% (Fig. 1). In contrast, in samples that were thawed in air the level of hemolysis exceeded 45%. Hemolysis in samples containing AFP was reduced in a concentration-dependent fashion to as low as 12% in the presence of AFP at 30–60 μg/ml (Fig. 1).

These samples contained plasma proteins. However, essentially identical results were obtained with red cell samples that had been washed with phosphate-buffered saline to remove plasma components (data not shown). In addition, enhanced cryopreservation was not due to additive effects of AFP and HES. AFP by itself did not protect red blood cells during freeze–thawing (data not shown).

Even in the samples thawed in a 45°C water bath, as the temperature approached the melting point there was a substantial decrease in the warming rate (see *Materials and Methods*) and, hence, a potential for ice recrystallization. However, without AFP, hemolysis was so low (Fig. 1) that any putative protection from AFP was difficult to detect. To improve the resolution of the system, the cryopreserved cells were diluted into phosphate-buffered saline after thawing, a treatment known to foster additional leakage of hemoglobin from red blood cells cryopreserved in HES (18, 19). With this treatment, enhanced preservation in the presence of AFP was apparent at all warming rates tested (Fig. 2).

Although AFP attenuated damage to red cells fostered by warming at suboptimal rates, Fig. 1 indicates that at up to 62 μg/ml, the protein did not confer complete protection from apparent recrystallization damage. The capacity of AFPs to inhibit recrystallization varies directly with protein concentration (13). Therefore, we tested the influence of a much larger range of AFP concentrations on the survival of cryopreserved red blood cells. Maximum protection was noted with AFP from 30 to about 150 μg/ml (Fig. 3). Surprisingly, further increase in AFP concentration led to less protection, and at the highest concentration tested (1.54 mg/ml), AFP fostered increased hemolysis. Thus, it appeared that, in addition to recrystallization inhibition, a second, undefined factor was governing cell recovery at high AFP concentrations.

To investigate the mechanisms for the protective and destructive actions of AFP, we used cryomicroscopy to observe the cell/HES mixtures during cooling and warming cycles. First, we compared ice crystal growth during slow warming in a sample without AFP to that noted in the

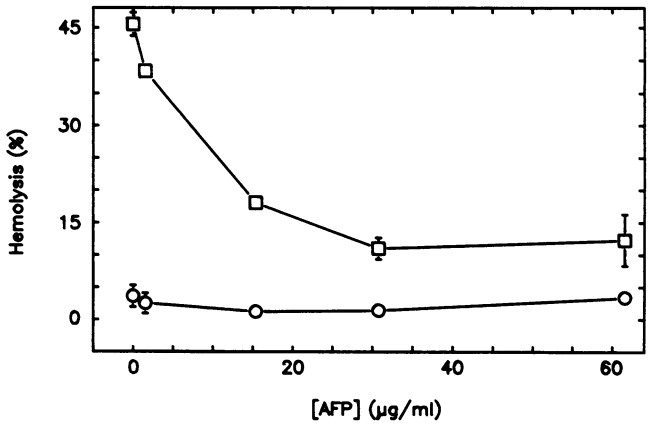

FIG. 1. Influence of AFP on hemolysis of human red blood cells cryopreserved in HES. One set of samples was thawed (rapid warming) by immersion in a 45°C water bath (○). An identical set was thawed (slow warming) in air at room temperature (□).

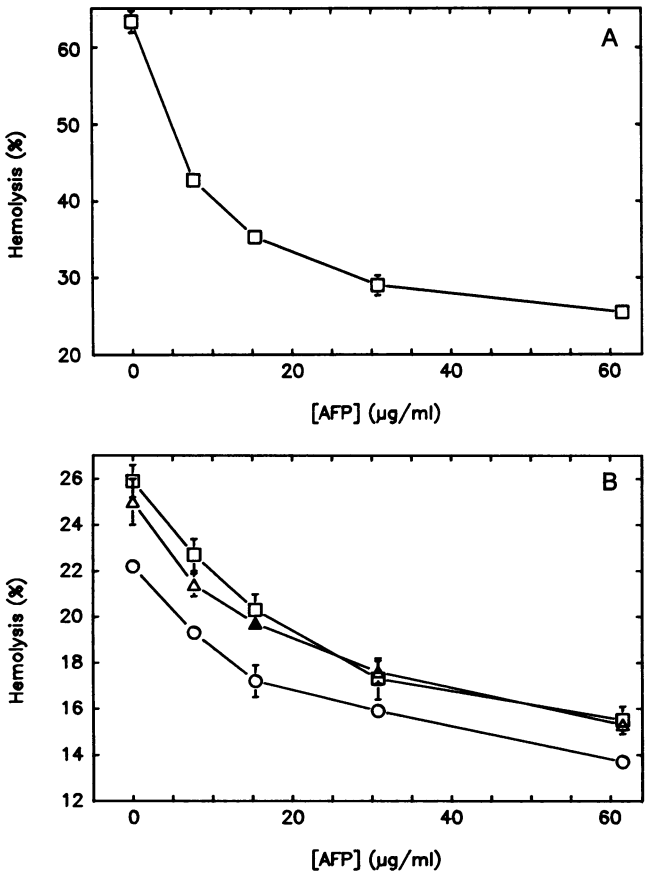

FIG. 2. Influence of AFP on hemolysis of human red blood cells cryopreserved in HES and diluted into phosphate-buffered saline after thawing. (*A*) Samples thawed in air at room temperature. (*B*) Samples thawed by immersion in water baths set at 23°C (□), 37°C (△), and 45°C (○).

presence of AFP at 62 μg/ml, which enhanced cell recovery (Fig. 1). Samples cooled rapidly (−600°C/min), with or without AFP, appeared optically clear at −133°C, with no ice crystals visible (Fig. 4). This appearance was maintained during warming, until the temperature reached about −11°C, at which point all samples almost instantaneously appeared grainy due to the formation of minute, visible ice crystals. AFP did not appear to affect the initial formation of ice crystals. With subsequent warming, in the absence of AFP,

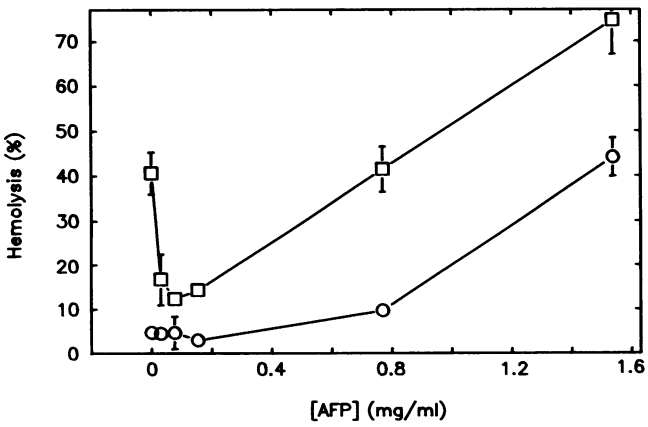

FIG. 3. Influence of a broad range of AFP concentrations on hemolysis of human red blood cells cryopreserved in HES. One set of samples was thawed (rapid warming) by immersion in a 45°C water bath (○). An identical set was thawed (slow warming) in air at room temperature (□).

there was a large increase in ice crystal size; just prior to the melt, the ice crystals were larger than, and completely obscured, the cells. After melting, there was extensive damage to the cells (Fig. 1). In contrast, in the presence of AFP at 62 μg/ml, ice crystal growth was attenuated (Fig. 4) and red cell survival was improved (Fig. 1).

Does this same mechanism apply to increased cell survival noted with AFP during rapid warming? Although the relative degree of ice growth without AFP was less than that noted during slow warming (Figs. 4*A* and 5*A*), there was still an obvious reduction in the size of ice crystals attained in the presence of AFP at 62 μg/ml (Fig. 5 *A* and *B*). Thus, the improved recovery for rapidly warmed cells (Fig. 2*B*) also correlated with AFP-induced inhibition of ice crystal growth.

Given this mechanism, one would expect that higher concentrations of AFP should confer greater protection, rather than being damaging. Earlier workers (20) noted that when red cells were cooled slowly (1°C/min) in the presence of 20% (vol/vol) glycerol and antifreeze glycoproteins at 40 mg/ml, the cells were mechanically damaged by AFP-induced spicular ice crystal growth. In our rapidly cooled samples, we could not detect an effect of AFP, at any level tested, on the appearance of samples prior to warming (Fig. 4). All samples were transparent and glassy, with no visible indication of ice crystal formation.

Furthermore, during both slow and rapid warming the presence of AFP at 1.54 mg/ml almost completely inhibited ice recrystallization in regions of the samples devoid of cells (Figs. 4 and 5). Alone, this property should have led to even greater cell recovery. However, under these conditions, in the regions comprising the cells and adjacent areas there was growth of massive, destructive ice crystals. This was especially obvious near the end of the slow warming protocol. When the bulk of the acellular region had melted, each position that formerly contained a cell was now occupied by a large ice crystal, which was angular and jagged in appearance (Fig. 4). This same pattern of cell-associated ice crystal growth (Fig. 4) and earlier melting of the acellular region (data not shown) occurred, but to a lesser degree, during rapid warming.

Another, perhaps related, observation with these samples was that near the end of the warming cycle small, bipyramidal ice crystals were interspersed among the cell-associated ice crystals (Fig. 4). These small crystals were similar in appearance to those that are formed when ice crystals are grown in the presence of AFP during cooling (e.g., ref. 6). In the current study, it appears that the plasma proteins—perhaps aggregates formed due to the salting-out action of HES on the proteins—were the focal point for formation of the bipyramidal ice crystals. When red cells were washed with phosphate-buffered saline to remove plasma components prior to mixing with HES and cryopreservation, all events were the same as those noted above with unwashed cells, except that these crystals were not seen during warming (data not shown).

DISCUSSION

Recrystallization of ice can be involved with damage to a variety of frozen and vitrified biological systems (e.g., ref. 21). As Knight, Duman, and coworkers (7, 9, 11) have proposed, freeze-tolerant organisms may be susceptible to this perturbation, either as they remain in the frozen state during long-term bouts of cold or as they thaw during warm periods. In conventional, *in vitro* cryopreservation, cooling is often rapid enough that many small ice crystals are formed. These tend to grow during the thawing cycle unless rapid warming is employed (21). Recrystallization can be even more problematic for attempts to preserve biomaterials by vitrification. In this case, sufficiently high cryoprotectant

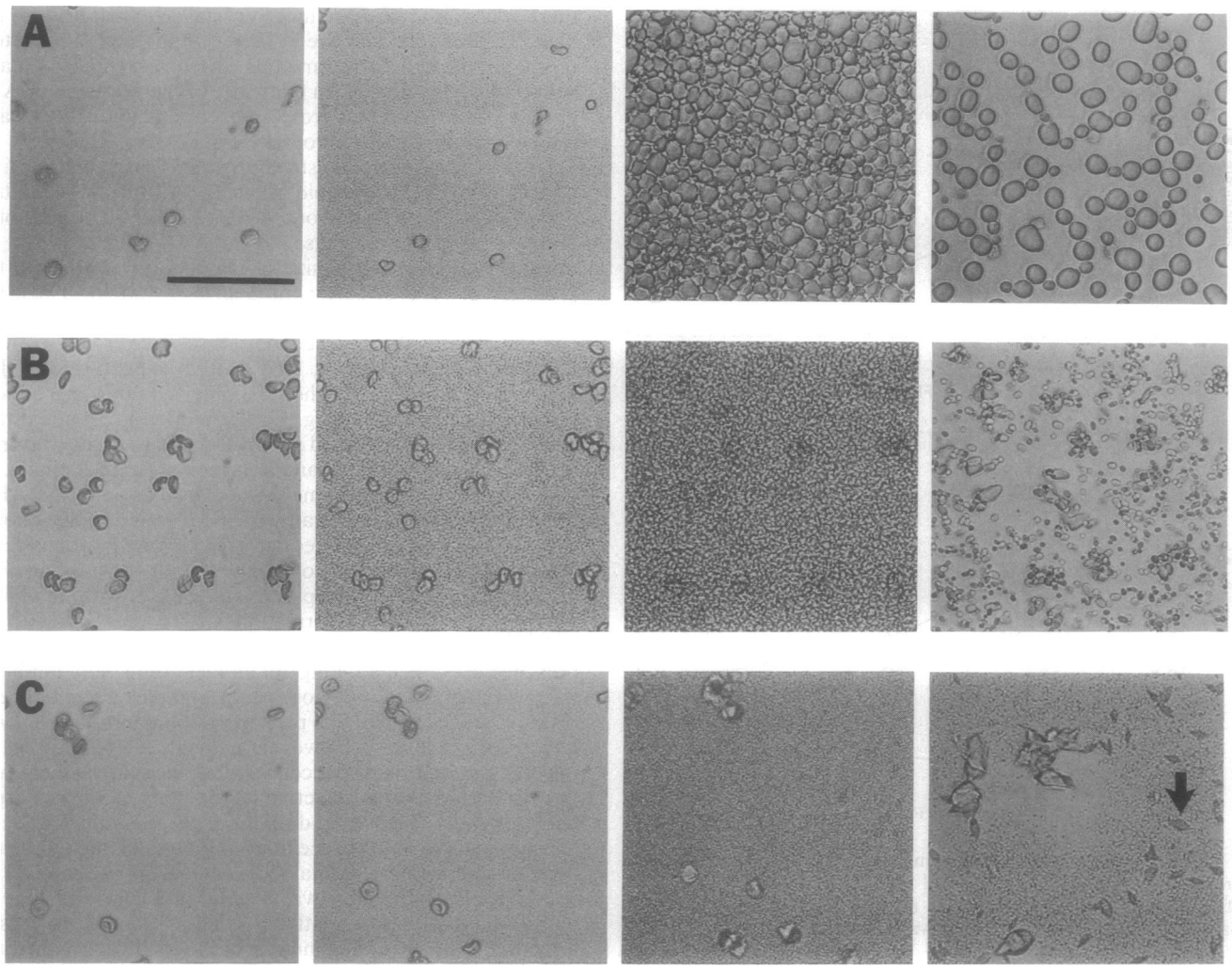

FIG. 4. Cryomicroscopic observations of the influence of AFP on ice crystal growth during cooling and slow warming. These experiments mimic the freeze–thawing conditions in the studies described in Figs. 1 and 3, when the samples were thawed in air at room temperature. Cooling and warming rates are given in *Materials and Methods*. Samples were prepared without AFP (*A*) or with AFP at 62 μg/ml (*B*) or 1.54 mg/ml (*C*). The micrographs, from left to right in each row, were taken at −133°C (before warming) and at −11 and −4°C during warming, and at the melt. Each panel in a given row represents the same initial field of view. The apparent shift in positions of cells is due to the slight lateral movement of the cryostage during the warming. The arrow for the sample containing AFP at 1.54 mg/ml, during the melt, indicates a bipyramidal ice crystal that is referred to in the text. (Bar = 50 μm.)

concentrations and cooling rates are used to prevent the formation of ice crystals during cooling (e.g., ref. 22). However, during warming the samples can devitrify, and the minute ice crystals formed can readily recrystallize. In fact, one of the major impediments to the practical, broad application of vitrification to biopreservation is the inability to rewarm samples rapidly enough to avoid devitrification and subsequent ice crystal growth (21, 22).

Our model system of red blood cells cryopreserved in HES undergoes "apparent vitrification"; our inability to discern visible ice crystals in samples cooled to −133°C does not necessarily mean that there were not crystals too small for

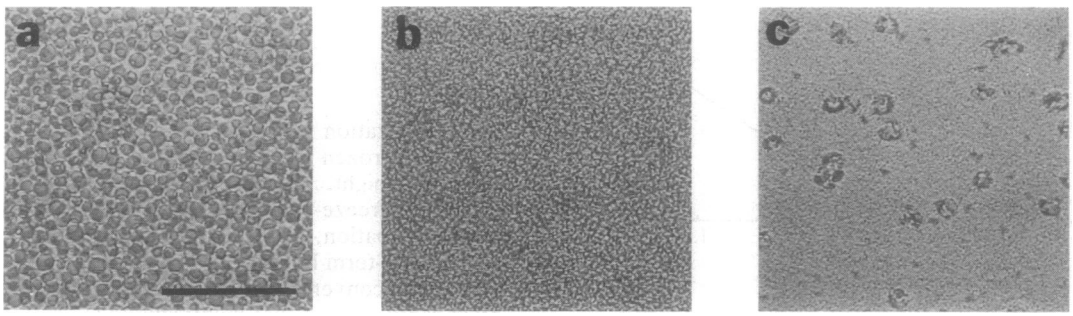

FIG. 5. Cryomicroscopic observations of the influence of AFP on ice crystal growth during cooling and rapid warming. These experiments mimic the freeze–thawing conditions in the studies described in Figs. 1 and 3, when the samples were thawed in a 45°C water bath. Cooling and warming rates are given in *Materials and Methods*. Samples were prepared without AFP (*A*) or with AFP at 62 μg/ml (*B*) or 1.54 mg/ml (*C*). Micrographs were taken during warming, just prior to the beginning of the melt (*ca.* −1°C). (Bar = 50 μm.)

detection. However, more importantly, this model is conducive to extensive recrystallization during warming, an effect that is magnified during slow warming. We have documented that AFP attenuates this ice crystal growth and that such inhibition correlates with the improved cell recovery noted with the protein.

This correlation holds even with the most rapid warming protocol used in this study. It is difficult to achieve greater warming rates and still avoid high-temperature damage to the red blood cells (18). Because of this compromise, recrystallization damage may be common in many cryopreservation protocols, particularly those employing relatively rapid cooling rates (see ref. 21). AFP could be a useful tool to investigate this possibility and may have further applications in enhancing cell survival. However, the protein's action is most likely limited to the extracellular fraction of the sample because the size of the protein (3300 Da) probably precludes penetration (without the use of membrane permeabilization techniques) into the cytosol. Further, there may be instances in which extracellular ice recrystallization does occur but is not the major limiting factor in cell recovery.

From our experiments, it is clear that both the protective and destructive effects of AFP on cryopreserved red blood cells are mediated through the influence of the protein on ice crystal growth during the warming cycle. In contrast, Rubinsky *et al.* (23) reported that the addition of high concentrations (>20 mg/ml) of antifreeze glycoprotein improved recovery of cryopreserved embryos, without an observable effect of the glycoprotein on ice crystals. However, their model system was quite different from ours. First, they both cooled and warmed the embryos in an apparent vitrification solution at 1700°C/min. Without the antifreeze glycoproteins, they noted no recovery of embryos, even though there was no visible formation of ice crystals at any stage of the process. They attributed the protective effects of the antifreeze glycoproteins to an as yet undefined, direct membrane-stabilizing effect of the carbohydrate moiety. Addition of an AFP (50 mg/ml), which had no constituent carbohydrate, conferred no protection. Together, their results and ours confirm that there must be ice crystal growth in the system to mediate the protective or destructive effects of nonglycosylated AFPs. We propose that, if used as described for our model system, the antifreeze glycoproteins, which are also known to inhibit ice recrystallization (12), would provide similar results.

The most surprising observation of the current study was that AFP at 1.54 mg/ml almost completely inhibited recrystallization and yet enhanced damage to the cryopreserved red cells. The mechanism by which AFP induces ice crystal growth around the cells during warming is not known (Figs. 4 and 5). Nor do we know how the bipyramidal ice crystals form in the extracellular region (Fig. 4), and whether these two events are mechanistically related. However, some observations on the cell-associated ice provide a basis for speculation that may be of use for future mechanistic investigations. First, the apparent volume of the ice crystal formed is much greater than could be accounted for solely by water derived intracellularly. Therefore, water molecules must be recruited from the extracellular ice crystals. AFP is thought (11–13) to inhibit migratory recrystallization because it adsorbs to the ice and blocks the addition of water molecules to the growing, more energetically favorable crystals. However, the migration of water molecules away from the smaller, thermodynamically less favorable crystals could still arise if there were a sink for these molecules. The regions around cells and protein aggregates appear to provide this sink. Second, the bipyramidal shape of the extracellular crystals and the angular, jagged appearance of the cell-associated crystals noted in the current study suggest that the AFP is incorporated into the ice crystals (5, 6). Also, we have observed large, apparently single, bipyramidal ice crystals growing around some red cells (unpublished observation). Thus, it may be that the AFP is bound to surfaces and for some unknown reason then serves as a focal point for ice crystal growth during warming.

In conclusion, we propose that there is a delicate balance between AFP-induced enhancement of cell preservation and AFP-induced enhancement of cell damage and that this balance hinges on the degrees of inhibition of ice recrystallization and of preferential growth of ice around the cells. Optimization of the practical benefits of AFP for cell cryopreservation will depend on understanding the mechanisms that govern this balance. However, at a minimum, it appears that empirical testing of AFP as an adjunct cryoprotectant should lead to improved protocols for cryopreservation, especially in rapidly cooled and vitrified systems. Finally, our *in vitro* results, at least indirectly, support Knight and Duman's (11) suggestion that the capacity of AFPs to inhibit ice recrystallization could be functionally important in freeze-tolerant organisms.

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