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Research Progress Report

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U.S. Naval Medical R & D Command

Contract No. N00014-90-C-0053

November 9, 1990

CRYOPHARM

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RESEARCH PROGRESS REPORT

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CRYOPHARM CORPORATION

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SUMMARY

The research described in this report has been supported in part by a three year basic research contract from the U.S. Naval Medical Research and Development Command, which began on May 15, 1990. This report summarizes our basic and clinical research efforts since September 30, 1990, the date of Cryopharm's last progress report.

As described in our September progress report, Cryopharm's 1990 milestones are twofold: 1) to initiate clinical safety and circulation studies of lyophilized autologous red cells in normal volunteers, and 2) continued basic research to produce overall improvements in cell properties and in vivo survival. The Basic Research section of this report outlines our progress in studying the total cation content and osmotic stability in saline of lyophilized reconstituted human red cells. Using stractan density gradients, we have identified fractions of recovered cells that exhibit improved osmotic stability and cation levels over our earlier formulations. We plan to implement the improved buffers and processing steps used to produce these higher quality cells during the upcoming clinical studies scheduled for January-March 1991.

The Clinical Research section outlines our recent low dose, autologous safety tests using lyophilized reconstituted red cells in normal adult male volunteers. These studies were conducted at the St. Elizabeth's Hospital of Boston, as approved by the hospital Institutional Review Board. In September-October 1990 we successfully conducted 51-Cr studies using two volunteers. Each volunteer received 19 ml and 25 ml, respectively, of packed, lyophilized reconstituted autologous red cells. The volunteers were carefully monitored over the initial 24 hours post-infusion, and daily for a total of 5 days post-infusion. Follow-up examinations were conducted after one week post-infusion. Both volunteers exhibited no changes in vital signs or any adverse side effects. Cultures of each blood sample were negative for microbial growth, indicating that our processing yielded sterile cell suspensions for transfusion. This constitutes a first validation of our sterile lyophilization container and the docking ports used to introduce rehydration and wash solutions.

Although we knew that the average in vitro osmotic stability of lyophilized human red cells in saline was lower than fresh red cells (see Appendix I and Notes), we expected to observe a more stable sub-population of perhaps 10-20% of the injected dose circulating in vivo. The results of the initial two clinical studies indicate that fewer cells (2-4%) appear to survive the initial few hours post-infusion. These results, confirmed by whole body radiation scans of the second volunteer, suggest that initial intravascular lysis is followed by splenic sequestration of the remaining intact cells and removal of free hemoglobin in the liver.

As described in the Basic Research section, we have identified conditions that yield cells with significantly higher osmotic stability than were available for the first clinical studies. In the Future Plans section, we describe our plan to implement these newer formulations for upcoming clinical studies in early 1991.

RESEARCH REPORT

In Appendix II we show the Milestones Chart taken from our September 1989 funding proposal. In the September 1990 progress report we addressed many of the listed Year I milestones. As discussed in the September report, we cannot use pig red cells as a viable in vivo circulation model. This progress report will first describe our ongoing basic research to improve the quality of lyophilized reconstituted human red cells. In the Clinical Research Section, we then discuss our first clinical studies, which due to the lack of a viable animal model constitute our initial in vivo research with lyophilized red cells.

I. Basic Red Cell Research

The focus of red cell basic research during 1990 is twofold: to devise improved buffers with respect to preservation of the ion balance and osmotic stability of the cells, and to streamline the post-rehydration washing. As described in this section, we have developed a second generation of buffers (not yet used in clinical tests) that yield cells with improved total cation levels and osmotic stability in vitro. Improved handling steps have also significantly raised the cell indices (MCV, MCH, MCHC) of lyophilized red cells (see Appendix I for a comparison between the initial clinical studies).

Buffer Formulation Research

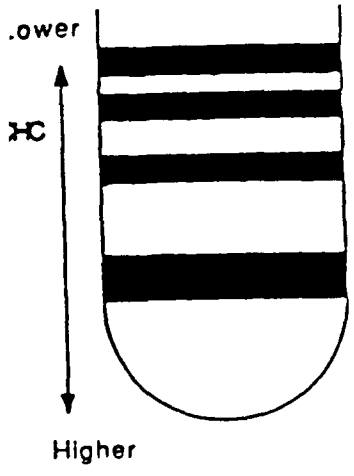
To produce the second generation buffers, we examined two parameters: the concentration of cryoprotectant and the concentration of added salts. Since our current formulations produce lyophilized reconstituted human red cells with 28-32% osmotic stability in saline, versus 98-100% for fresh cells (see Notes to Appendix I for the osmotic stability assay), we looked at lower solute concentrations to try to avoid "solute loading" by the cells during processing. Our hypothesis is that excess solute internalized within the cells leads to an influx of water during rehydration that causes cell swelling and lysis.

In order to quantitate the effects of different solutions on the total cell population, we centrifuge red cells through discontinuous stractan density gradients, using a published technique (1). In Figure 1 an illustration of the density profile achieved by a stractan gradient is shown. We find that the most dense cells sediment to density levels III and IV (around 1.08-1.10 g/ml) shown in Figure 1. This is true of both fresh human red cells and a percentage of lyophilized reconstituted human red cells (see Figure 1). Lighter cells are found in Fractions I and II, and contain cells with low density, high MCV (swollen), low MCH, and low MCHC. This is supported by light microscopy, which shows that Fractions I and II contain swollen and fused cells, and hemolyzed cell debris.

In Graph 1 we show a significant correlation between the osmotic stability in saline, the MCHC, and the stractan density fraction of lyophilized reconstituted human red cells. As shown in this graph, recovered cells from the more dense gradient fraction III also have higher MCHC and osmotic stability. Some of the more dense samples tested have quite improved osmotic stabilities in the 80-90% range. This contrasts with our two clinical test samples, in which the average osmotic stabilities of the infused samples were 28% and 32%, respectively (Appendix I).

FIGURE 1

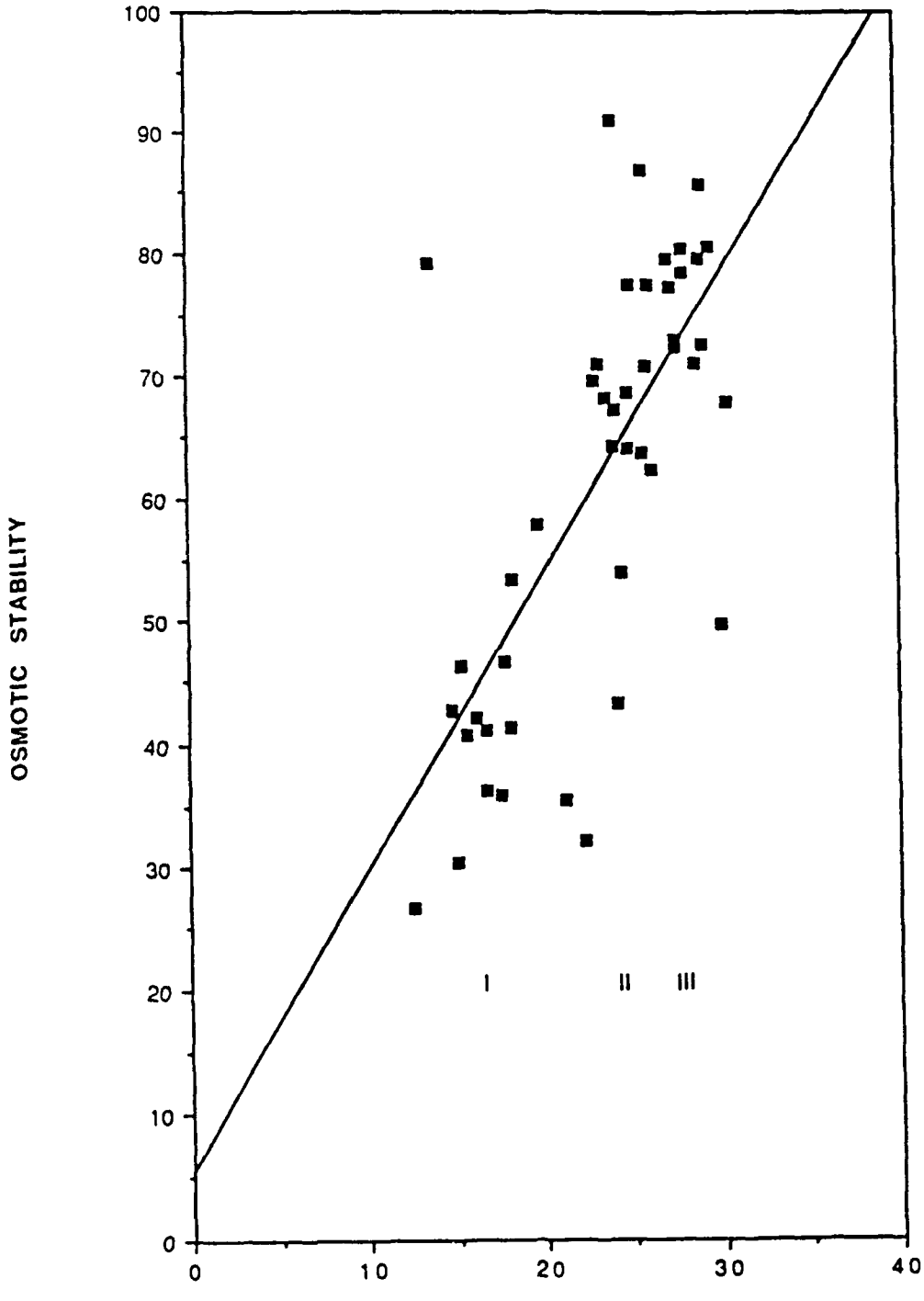
Illustration of Stractan Density Gradient and Current Yield of Lyophilized Reconstituted Human Red Cells in Each Density Fraction.



		Percent Recovered RBCs in Each Fraction	
		Lyophilized Reconstituted	Fresh Controls
I	<1.04 g/ml	16.3	0
II	<1.06 g/ml	0	0
III	<1.08 g/ml	67.5	94.1
IV	<1.10 g/ml	16.2	5.9
V	>1.10 g/ml	0	0

GRAPH 1

CORRELATION OF OSMOTIC STABILITY AND MCHC



Our goal is to increase the proportion of lyophilized reconstituted red cells that have normal stractan sedimentation (i.e., fractions III-IV) and the associated high MCHC and osmotic stability in vitro. The following two Tables illustrate the various conditions that we have found to influence the resulting density of lyophilized reconstituted human red cells. In Table 1 we show that "solute loading" is evidently a significant problem in terms of the resulting cell density and associated osmotic stability. It appears that a successful lyophilization buffer will require compromises between the need for cryoprotectants, and the need to minimize solute loading by the cells during processing. In Table 2 we further show that agitated mixing of the packed red cells with any lyophilization buffer is more damaging than a more gentle physical mixing. We presume that agitated mixing is detrimental due to the generation of shear forces in the solution (this may be further improved by studying the viscosity effect introduced by the lyophilization buffer). This interpretation agrees with published studies showing that lysis of fresh human red cells is achieved by shear forces generated during mixing in solutions of increasing viscosity (2).

Table 1.

Stractan Gradient Analysis of Lyophilized Reconstituted Human Red Cells Produced Using Buffers With Different Solute Concentrations.

Lyophilization Buffer Cryoprotectant Concentration	Added Salts		% Recovered Cells in III-IV Stractan Density Fractions
	Lyoph. Buffer	Rehyd. Buffer	
Low	-	-	67.6
Low	+	-	48.2
Low	+	+	41.9
High	+	-	39.0
Low	-	+	37.7
High	-	-	36.7
High	-	+	32.1
High	+	+	31.4

The data in Table 1 represent single experiments using different units of refrigerated blood as starting material. Although standard errors are not available, the ranking of single stractan data points does indicate a clear trend. Buffers with the highest cryoprotectant levels and added salts (i.e., high solute solutions) produce fewer cells in the desired normal density range. This suggests that "solute loading" of cells is exacerbated in high solute

solutions, and this leads to more cells in the lighter I-II fractions, indicative of swollen or lysed cells.

Table 2.

Effects of Solute Concentrations and Mixing on Cell Density of Lyophilized Reconstituted Human Red Cells.

Lyophilization Buffer Cryoprotectant Concentration	Added Salts Lyoph. Buffer	Added Salts Rehyd. Buffer	Blood/ Buffer Mixing	% Cells in Stractan III-IV Fractions
Low	-	-	Vigorous	21.7
Low	-	-	Gentle	67.6
High	-	-	Vigorous	5.2
High	-	-	Gentle	36.7

From Table 2 it can be seen that a more gentle mixing of packed red cells with a lyophilization buffer containing lower levels of cryoprotectant yields a final population of lyophilized reconstituted cells having over half (67%) of the recovered cells in the high density fractions III-IV of the stractan gradient. These cells will also show a higher MCHC and in vitro osmotic stability, as previously shown in Graph 1.

Total Cation Content and Osmotic Stability

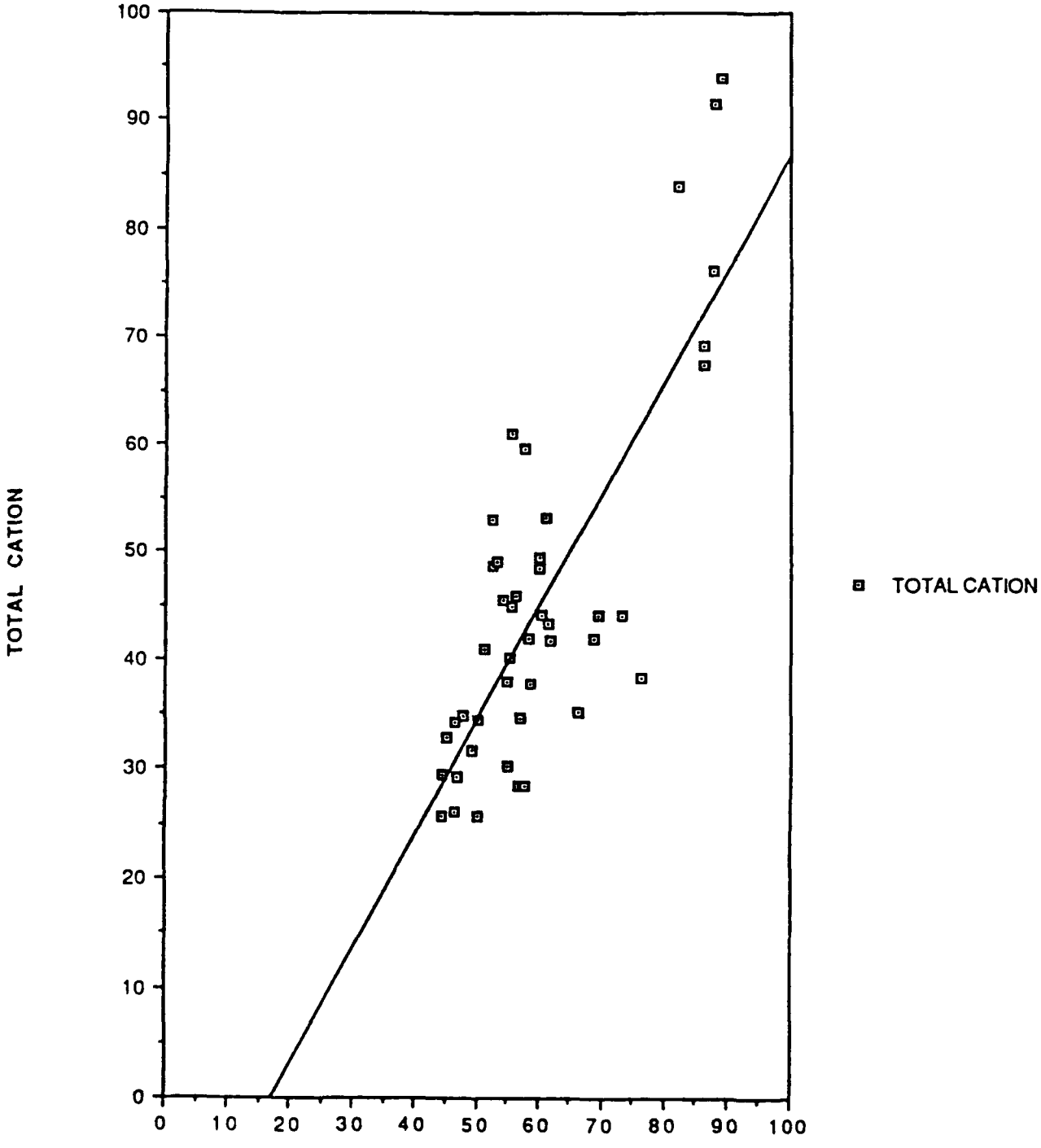
In addition to the buffer solute concentrations and the mixing protocol, we find that restoration of the cation levels in lyophilized reconstituted human red cells is also important to in vitro osmotic stability. As shown in Graph 2, we find a direct correlation between osmotic stability in saline and total cation content (mEq/l) in the recovered cells. We assay for cation contents using a standard flame photometer method (3). Fresh human red cells have total cations in the range of 100-120 mEq/l. Our most recent lyophilized samples, using the improved buffers and mixing procedure, are in the 30-40 mEq/l range in total cation, with some preparations as high as 80-90 mEq/l. Clinical testing of cells treated accordingly will determine if cell circulation is also improved.

Antioxidants

As discussed in the September progress report, we have researched several antioxidants to minimize damage to the hemoglobin during lyophilization. Our hypothesis is that oxidized hemoglobin produced in the dry state can yield hemin as a degradation

GRAPH 2

TOTAL CATION VS OSMOTIC STABILITY AFTER INCUBATIONS



product. Electron spin resonance studies of lyophilized red cells conducted with Dr. Matthew Platz of The Ohio State University have confirmed the presence of hemin in our reconstituted human red cells (these samples were prepared using our first generation buffers). The improved second generation buffers will incorporate antioxidants, which are not currently added to our buffers, and will minimize oxidative damage to the hemoglobin and other cell components. We suspect that damaged hemoglobin can in turn catalyze further damage to the cell membrane, which may affect cell stability. Our second generation of buffers with antioxidants will address this issue.

Streamlining of the Wash Steps

Concurrent basic research efforts are designed to minimize the number of wash solutions and the time needed on the Cobe cell washer for each step. In order to reduce the total number of wash solutions, Basic Research is now evaluating the sequence of wash buffer conditions presently used, with the goal of combining some of the existing wash buffers. Reduced numbers of wash steps and reduced volumes of sterile liquids consumed will impact the economic and ease-of-use issues of this technology.

Cryopharm is also evaluating improved procedures for rehydration of the dried cells. Basic research efforts have shown that minimized mechanical stress during processing can significantly improve the quality of the final washed cells. We plan to introduce an improved shaking protocol during rehydration that will lessen shear stress on the cells.

II. Process Development and Container Design

Process Development

In the process development area, our research is driven by two key goals: to minimize the potential for cell damage during processing, and second to define the optimal freezing and lyophilization conditions. These efforts should lead to improved yields of higher quality cells.

Our efforts to minimize processing damage to the red cells are focused on the mixing and reconstitution/wash conditions. We are currently evaluating a more gentle protocol for mixing cells with lyophilization buffer, and we have observed a significant improvement in the red cells indices (mean cell volume, mean cell hemoglobin content) with more gentle mixing. This result has been described in Table 2. The improved mixing protocol avoids vigorous shaking, and this appears to minimize loss of hemoglobin from the cells (hence the mean cell volume and mean cell hemoglobin increase to within the normal range for fresh human red cells). We plan to include this new mixing for the 1991 clinical studies.

Process damage to the cells can also be reduced by more gentle swirling during reconstitution, and using shorter spin times on the Cobe cell washer. We plan to introduce shorter Cobe wash times for the upcoming clinical studies. Shorter spin times will reduce the overall process time, and avoid excessive packing of the cells during centrifugation.

During the remainder of 1990, the Process Development Group will also focus on freezing conditions and lyophilization cycle research. The group has been studying various freezing rates and end-point temperatures using shelf lyophilizers. The goal will be to determine a freezing protocol that is rapid and effective. Other studies will focus on the actual drying cycle, which involves a primary and secondary phase. A range of shelf temperature set-points for each phase will be examined, and the length of time that each drying phase should require. Again, these experiments will try to define optimal conditions for drying that will minimize the overall cycle length. Our current 10-day cycle is deliberately conservative to ensure thorough drying for the clinical studies. We believe this time can be shortened to 4-7 days, looking ahead to maximizing lyophilizer throughput for process scale-up.

Container Design

Cryopharm's first working prototype lyophilization container is constructed of rigid thermoformed plastic. Product is presently dried via an open port. We plan to study other designs that incorporate a particle barrier to preclude cross-contamination between units. In addition, the positions and sizes of connecting tubing and ports must be modified to simplify filling of the container. Finally, a longer term design issue involves sealing of bags while under vacuum and long-term shelf-life studies in the bags.

III. Clinical Research

Purpose of Initial Studies

Cryopharm's first generation of buffers produce lyophilized reconstituted human red cells with reduced osmotic stability (~30%) in saline, relative to fresh red cells in saline (98-100%). The autologous whole blood stability of 51-Cr labeled lyophilized cells prepared with the same buffers is higher (~70-80%). We reasoned that such lyophilized cells, if tested in vivo, would contain a subpopulation that could survive for some period in circulation (perhaps up to 10-20% of the recovered cells distributing into several clearance phases with distinct clearance half-lives). In both studies higher doses of 51-Cr were used to identify subpopulations of cells that might exhibit prolonged circulation. The purpose of these studies was therefore to identify any subpopulations of circulating cells, their relative abundance, and ultimate fate using whole body scans.

First Clinical Study

The first clinical study was conducted at St. Elizabeth's Hospital of Boston during September. The attached summary chart (Appendix I) highlights some of the key in vitro parameters measured for the clinical lyophilized reconstituted red cells, compared to fresh red cells. This sample was prepared using our current "First Generation" lyophilization and reconstitution/wash buffers, and using our original mixing protocol to mix the cells and

lyophilization buffer. As shown in the summary chart, the red cell indices (MCH, MCV, MCHC) were below normal in this sample, and the osmotic stability of 28% is significantly lower than for fresh red cells.

In this first study, only half of the required 51-Cr was used to label the red cells, which prevented us from conducting whole body scans to visualize where in the body the label was accumulating. This deficiency was corrected in the second study.

The cell circulation results of this first study are shown in Table 3 (all data are from 30 minute counts of peripheral whole blood samples). Due to difficulties with the sampling catheter, the planned 12 minute and 15 minute blood samples could not be obtained. As seen in Table 3, however, rapid clearance within the first 5 minutes post-infusion prevent a meaningful back-extrapolation of the time-zero dose. Therefore we use the theoretical time-zero value to estimate cell survival. In this experiment the majority of the labeled cells are rapidly removed from circulation, such that within 4 hours post-injection some 3% of the theoretical total dose is still circulating. This level does not drop significantly between 4 hours and 96 hours post-injection. Due to our inability to scan the volunteer's body, we could not determine the fate of the removed cells. We suspected clearance by the spleen, since little radioactivity appeared in the urine (9.9% of the total injected counts were recovered in urine during the first 24 hours post-infusion). However, the low dose was insufficient to saturate circulating haptoglobin, hence the actual clearance mechanism remained ambiguous. The injection of this volunteer produced no changes in vital signs, indicating that the dose (19 ml of packed cells) did not present a safety threat.

This study revealed some 2% of the injected label still in circulation at 96 hours post-injection. We cannot say whether this remaining level represents intact red cells. In the second study we emphasized the whole body scans, to try to determine the fate of the injected cells.

Second Clinical Study

The second clinical study was conducted as described for the first study. As shown in Appendix I, we were able to significantly improve the red cell indices in the sample using a gentler mixing protocol, although the osmotic stability was only marginally increased. This study therefore addresse the issue of whether improved cell indices could result in improved cell survival. We believe that the improved cell indices are a direct result of the improved mixing procedure that reduces mechanical stress on the cells. In this study we again used the First Generation of buffers.

In Table 4 the cell circulation results of the second study are shown (these data are also 30 minute counts of peripheral whole blood samples). As in the first study, no changes in vital signs were observed. The initial value at 5 minutes post-infusion is lower than in the first study due to the longer time needed (some 10 minutes) to inject the larger, 25 ml dose of packed cells. The majority of these cells are also rapidly cleared from peripheral circulation. In Graph 3 we plot the estimated percent injected dose over the first 4 hours post-infusion for both clinical studies. Although there is some evidence for an improvement in short-term circulation (both phases have a 3.5 hour half-life), the amount of initial lysis requires a cautious interpretation.

TABLE 3

SUMMARY OF RESULTS CLINICAL STUDY 001-90

Time Point	Raw CPM	Background Corrected CPM	% Injected Dose
5 Min	289.8	165.7	21.0
7.5 Min	233.6	109.5	13.9
20 Min	185.8	61.7	7.8
30 Min	178.0	53.9	6.8
45 Min	174.5	50.4	6.4
60 Min	173.8	49.7	6.3
2 Hours	161.6	37.5	4.8
4 Hours	149.3	25.2	3.2
6 Hours	144.7	20.6	2.6
24 Hours	141.2	17.3	2.2
48 Hours	138.8	14.9	1.9
72 Hours	139.6	15.7	2.0
96 Hours	140.2	16.3	2.1

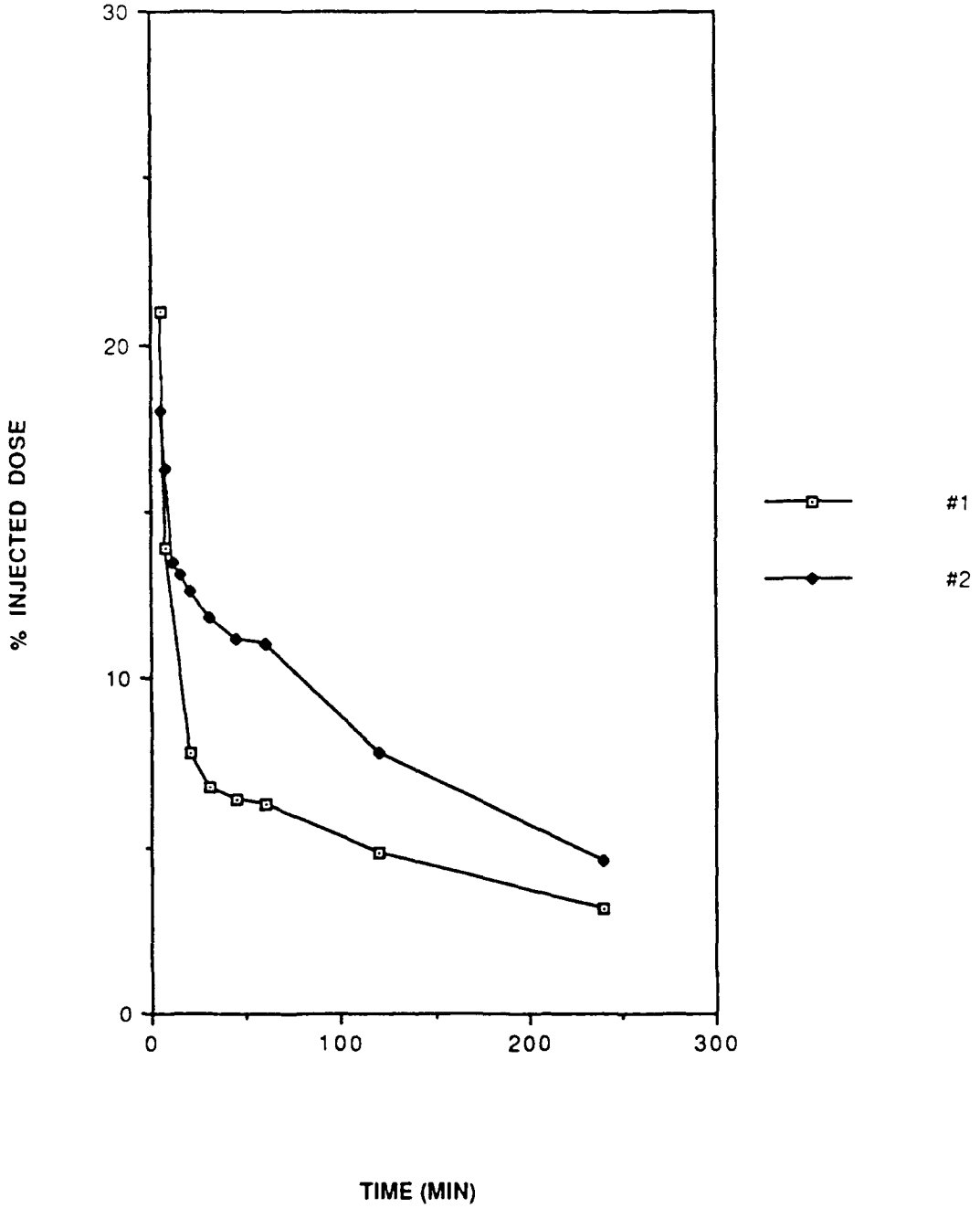
TABLE 4

SUMMARY OF RESULTS CLINICAL STUDY 002-90

Time Point	Raw CPM	Background Corrected CPM	% Injected Dose
5 Min	590.5	463.3	15.5
7.5 Min	496.6	369.4	12.3
12 Min	473.6	346.4	11.6
15 Min	481.6	354.4	11.8
20 Min	455.3	328.1	11.0
30 Min	434.6	307.4	10.3
45 Min	408.5	281.3	9.4
60 Min	410.9	283.7	9.5
2 Hours	327.6	200.4	6.7
4 Hours	242.4	115.2	3.8
6 Hours	217.7	90.5	3.0
24 Hours	193.6	67.2	2.2
48 Hours	182.3	55.8	1.9
72 Hours	182.9	56.5	1.9
96 Hours	179.5	53.1	1.8

GRAPH 3

GRAPH OF % INJECTED DOSE FROM 5 MIN-4 HR



In this study we were able to conduct whole body scans, and these photographs clearly show the majority of radioactivity in the liver, and not in the spleen. In Table 5 we show the ratios of 3 minute body scans taken over the spleen, liver, and precordium.

Table 5.

Ratios of Body Counts At 4 Hours and 24 Hours Post-Infusion.

Organ Areas Compared	4 Hours	24 Hours
spleen/precordium	1.5	2.4
liver/precordium	3.9	5.5
spleen/liver	0.4	0.4

With fresh 51-Cr labeled red cells, the organ ratios are typically 1:1:1 comparing spleen/precordium : liver/precordium : spleen/liver, due to the extensive blood flow through these organs. The data in Table 3 indicate that more 51-Cr is cleared from circulation by the liver, versus the spleen. This indicates that most 51-Cr clearance is likely due to removal of free hemoglobin/haptoglobin by the liver. In this second study, some 8% of the injected dose of 51-Cr was recovered during a 24 hour urine collection. The remaining label in the spleen may represent intact cells removed by splenic sequestration.

As in the first study, by 96 hours post-injection some 2% of the injected dose remains in circulation. Due to the low level of circulating label in these longer time points, it is not possible to establish whether the label represents intact circulating cells.

FUTURE PLANS

The purpose of these first clinical studies was to test our hypothesis that a subpopulation of lyophilized cells could remain in circulation. We estimated that perhaps 10-20% of injected cells would exhibit some level of circulation, based on the in vitro deformability and osmotic stability in saline and autologous whole blood. It appears from the clinical results that a smaller fraction of the injected cells, less than 10%, circulate briefly within the first 4 hours post-infusion. The significant lysis following infusion prevents a more accurate estimation, and prevents us from saying that any cells remain in circulation beyond the initial 4 hours.

In January 1990 we identified ion balance, osmotic stability, and oxidation damage

as important parameters for further research. We began to address these parameters in mid-summer, and this effort has produced the second generation of buffers that we intend to use for the next series of clinical studies. The second generation of buffers, to be introduced in January 1991, and ongoing process improvements to minimize processing damage to the cells, begin to address the cell stability issue. For example, using these improvements we now recover lyophilized reconstituted human red cells with an average osmotic stability of 50-60% in saline, versus the approximately 30% in the first clinical samples. Nevertheless, the central question remaining is to identify which cell properties are vital to cell circulation, and what levels will support in vivo cell survival.

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APPENDIX I

CLINICAL STUDY SUMMARY

CLINICAL STUDY PARAMETERS	FRESH RED BLOOD CELLS	LYOPHILIZED RED CELLS	
		CLINICAL STUDY 1	CLINICAL STUDY 2
Cell Indices (*)			
Mean Cell Hemoglobin	25.4-34.6 pg	13.9 pg	22.4 pg
Mean Cell Volume	80-100 fl	70.7 fl	89.0 fl
Mean Cell Hemoglobin Concentration	31-37%	19.6%	25.1%
Osmotic Stability	98-100%	28.0%	32.6%
ATP Levels	3.65-4.45 micromol/g.	2.82 micromol/g.	3.04 micromol/g.
Lactate Synthesis	6.87 $\pm$ 0.65 micromol/g/hr	9.77 micromol/g/hr	19.2 micromol/g/hr
Blood Type/ Crossmatch	Compatible	Compatible	Compatible
Buffers Used	Not Applicable	Generation 1	Generation 1

NOTES TO APPENDIX I.

(*) Cell Indices are defined as follows:

Mean Cell Hemoglobin (MCH): the average per cell Hb content, in picograms.

Mean Cell Volume (MCV): the average cell volume in femtoliters.

Mean Cell Hb Concentration (MCHC): the average per cell Hb concentration from MCH and MCV.

Osmotic Stability: the percent of starting red cells that remain intact in normal saline (0.85% w/v) at room temperature for 30 minutes.

ATP Level: the cellular ATP content in micromoles per gram of hemoglobin.

Lactate Synthesis: the rate of lactate production in micromoles/gram Hb/hour, which shows the relative metabolic viability of the cells.

Blood Type/Crossmatch: all samples are compatible with autologous plasma; i.e., they retain their original ABO/Rh type and do not bind the donor's own antibodies or complement. Aliquots of reconstituted cells are typed and crossmatched by the hospital blood bank prior to transfusion.

APPENDIX II. Research Milestones Chart From Cryopharm's September 1989 Research Proposal.

CRYOPHARM CORPORATION
RESEARCH MILESTONES CHART
FREEZE-DRIED RED CELLS

Project Activities	Current Status	Milestone	Projected Start	Projected Completion
Define Shelf Lyophilization Parameters: Define optimal temperature, pressure conditions. Evaluate sample configuration.	No defined cycle	Defined cycle worked-out	Year 1	Year 1
Evaluate Existing Reconstitution Protocol: Mixing and temperature conditions.	~70% initial yield	>80% initial yield	Year 1	Year 1
Optimize Product Properties:				
Cell yield (at infusion stage).	~35-40%	>50%	Year 1	Year 2
Residual moisture (in dry state).	~3%	~1%	Year 1	Year 2
Final product sterility (at infusion stage).	Not done	Demonstrated	Year 1	Year 2
Shelf Life:				
Refrigerated storage.	>10 months	>2 years	Year 1	Year 3
Room temperature storage.	~2 weeks	1-2 months	Year 1	Year 3
Evaluation of Enzyme Converted Red Cells.	Not done	Initial tests	Year 2	Year 2
In vivo Animal Circulation Studies:				
Pilot studies in domestic pigs.	Not done	Done	Year 1	Year 1
GLP quality studies in domestic pigs.	Not done	If pilot tests successful.	Year 2	Year 2
In vitro Animal Red Cell Studies: (Survey models if pig cells do not circulate)	Preliminary data in.	More samples for FDA.	Year 2	Year 2
Plastic Container Development.	First prototype	Developed.	Year 1	Year 1
Streamline Reconstitution and Washes.	Not done	Underway	Year 3	To be deter.
Phase I Clinical Trials of Lyophilized Cells.	Not done	File IND	Year 3	Continues...