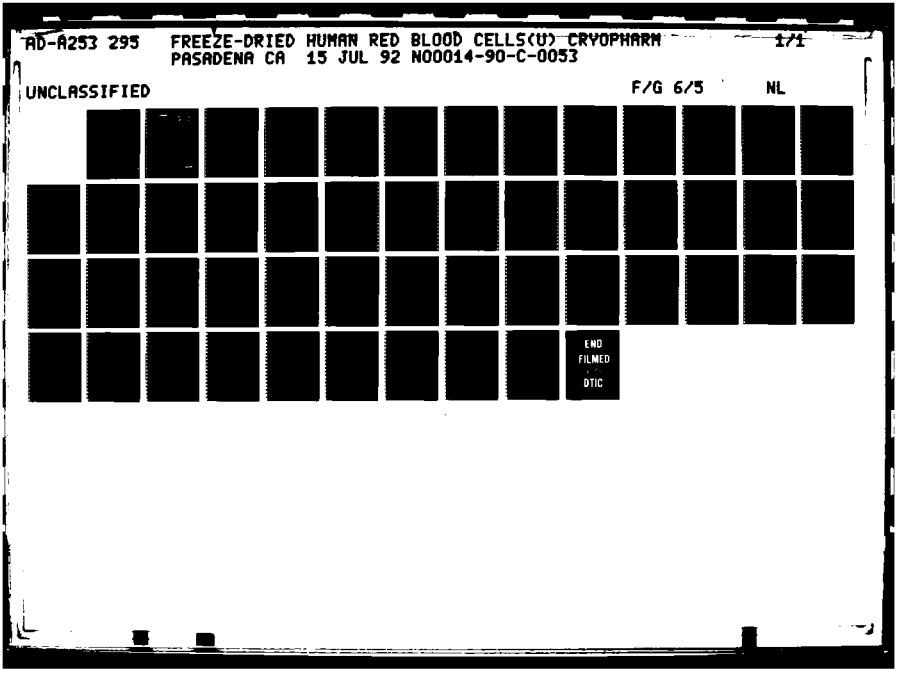
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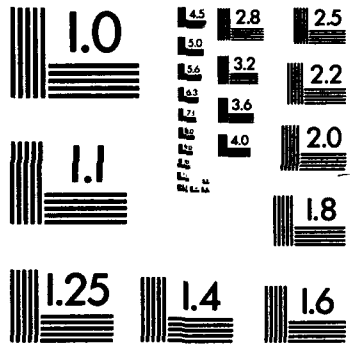
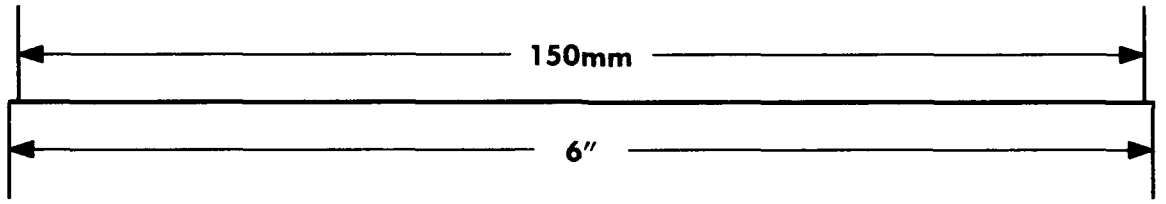




PHOTOGRAPHIC SCIENCES CORPORATION
770 BASKET ROAD
P.O. BOX 338
WEBSTER, NEW YORK 14580
(716) 265-1600

Research Progress Report

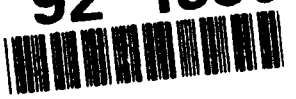
submitted to the

U.S. Naval Medical R & D Command

Freeze-Dried Human Red Blood Cells

Contract No. N00014-90-C-0053

July 15, 1992



CRYOPHARM
2585 Nina Street
Pasadena, California 91107

RESEARCH PROGRESS REPORT

SUBMITTED TO THE US NAVAL MEDICAL RESEARCH AND DEVELOPMENT COMMAND

FREEZE-DRIED HUMAN RED BLOOD CELL

CONTRACT NO. N00090-C-0053

CRYOPHARM CORPORATION

July 15, 1992

TABLE OF CONTENTS

SUMMARY

This research progress report focuses on CryoPharm's third clinical study and summarizes our progress in basic red blood cell research since our last progress report submitted to Naval Medical Research and Development Command on April 15, 1992.

As outlined in the summary report from April 15, 1992, a preliminary clinical evaluation of in vivo circulation of autologous, lyophilized and reconstituted human red blood cells was undertaken to establish a baseline level of in vivo performance of lyophilized red blood cells from both human volunteers and cynomolgus monkeys. The cell survival results demonstrated that reconstituted, lyophilized red blood cells remained in circulation in vivo for the same period as normal non-lyophilized red blood cells.

Since our last progress report we have obtained additional in vivo survival data from a total of four healthy volunteers. The design of the clinical study protocol followed accepted procedures for red cell in vivo survival studies. In all of the four volunteers , no changes in vital signs occurred after an infusion of a small dose of the autologous lyophilized red blood cells and no associated adverse side effects were observed during the study and through the final follow-up examination conducted one week post infusion.

In all four volunteers, peripheral blood samples were collected in citrate-phosphate-dextrose and adenine-1 (CPDA-1) for up to 6 days post infusion to measure the level of circulating chromium labeled red cells and to allow accurate estimation of the circulating half lives of the cells. Urine sample collection was also carried out during the first 24 hours post infusion for measurement of the levels of chromium clearance by the kidneys. Each volunteer was subjected to gamma imaging and external probe counts over the heart, spleen and liver at 4 hours and 24 hours post-infusion to determine the organ distribution of the radiolabeled reconstituted red blood cells.

The data from this clinical study clearly demonstrated that:

1. Chromium labeled reconstituted, lyophilized red blood cells have an average in vivo survival of 90% at 24 hours post infusion. This level of survival is comparable to that for fresh non-lyophilized red blood cells.

2. The mean circulation half life is about 25 $\pm$ 5 days. Mean half life of fresh red blood cells as measured by ^{51}Cr red cell survival is 28-30 days.This level of in vivo survival correspond to mean lifespan of between 115 and 120 days.

3. There was no sequestration of radiolabeled red blood cells into any of the major organs. This suggests that most of the biophysical properties that are known to influence trapping of red blood cells in vivo are not affected by our lyophilization process. Gamma imaging of the three highly vascularized organs of the body (heart, liver and spleen) show a uniform distribution of the reconstituted, lyophilized red blood cells. These distribution profiles are similar to that for normal non-lyophilized fresh red blood cells.

4. External probe counts taken over the heart, liver and spleen to determine the distribution of the lyophilized red blood cells at 4 hours and 24 hours post transfusion showed approximately equal proportions over each organ. These results are the same in all four volunteers studied. This agrees with the results of gamma imaging technique and are confirmatory of a normal cell distribution profile.

5. The level of loss of radioactive chromium in the urine via the kidney is less than 5% in all of the four volunteers and is well within the acceptable limit for fresh non-lyophilized red blood cells. Again, this is a reflection of a normal clearance of released chromium by the kidney.

6. No significant changes in vital signs (body temperature, blood pressure and heart rate) were observed in any of the four volunteers during and 5 days after the study.

7. In none of the volunteer did we detect any significant binding of autoantibodies to autologous reconstituted , lyophilized red blood cells, using standard clinical crossmatching procedures. This result demonstrated that red cell surface properties were not altered by our lyophilization procedure.

8. Small infusions of lyophilized red blood cells into autologous donors did not appear to interfere with normal blood chemistry.

9. Levels of metabolic intermediates in lyophilized red blood cells (such as adenosine 5'triphosphate, lactate and 2,3-diphosphoglycerate) are comparable to that of fresh red blood cells. Oxygen carrying capacity of lyophilized red cells are also similar to that of fresh cells. For example the oxygen tension at which the hemoglobin is half saturated is 25mmHg for lyophilized red blood cells compared to 27mmHg for fresh red blood cells.

We believe that the above in vivo survival data show for the first time that human red blood cells can be lyophilized, reconstituted and infused into healthy volunteers with retention of normal physiological properties. Further studies will be needed to begin to address the dose build up and safety issues.

UPDATE OF PROJECT STATUS RELATIVE TO 1989 MILESTONES

Cryopharm submitted its original research proposal on lyophilized human red blood cells in September, 1989. In that proposal we included a chart of research milestones and a copy of that chart is included in this report, Table 1. In our Progress Reports of November 1991, January 1992 and April 1992, we outlined all our progress to date with respect to the proposed projects and we showed that most of the proposed studies have been completed at the specified projected dates.

Our final 1989 milestone proposed the filing of an IND for Phase I clinical trials at the end of Year 3 (May 1993). We believe that our project remains on target. We have already successfully conducted low dose autologous human red blood cells in vivo survival studies with four healthy volunteers in collaboration with the Department of Biomedical Research at Tufts University School of Medicine. These studies have been conducted with the approval of the appropriate Institutional Review Board. The design and dosage of these studies parallels what would be used in any Phase I clinical trial. We have demonstrated the safety of the lyophilized product in four healthy volunteers as reported in the present Progress Report. With our present successful clinical trial, we anticipate filing for an IND at the projected date.

RESEARCH PROGRESS REPORT

Background

In the April 15, 1992, Progress Report we reported that Cryopharm's basic red cell research had developed an improved lyophilization buffer formulation that allowed us to successfully lyophilize human red blood cells. The reconstituted cells exhibited properties that are similar to that of normal, fresh non-lyophilized red blood cells . In addition, autologous chromium labeled reconstituted lyophilized red blood cells were infused into a healthy volunteer during Cryopharm's third clinical study in April 1992. Preliminary reports from this single volunteer was reported in our April Progress Report along with in vivo survival data from Cynomolgus monkeys. These initial clinical results encouraged us to increase the human survival study to include an additional three volunteers.

Human Clinical Study

The clinical study protocol that was used in the present in vivo survival study is included in this report for reference. This protocol describes in detail the study procedures and the methods used for labeling of reconstituted, lyophilized red blood cells with radioactive isotope, (sodium chromate -^{51}Cr), collection of urine and peripheral blood samples, gamma imaging, and calculation of whole blood volume and percent injected dose in circulation. Criteria used for volunteer selection and monitoring

of vital signs are also included. Briefly, this study was designed to determine if human red cells could be lyophilized, reconstituted and then infused into healthy volunteers without any undue risks to the volunteers. After admission into the study protocol, the volunteer was phlebotomized and 450mL of blood was collected in a standard blood bank bag containing citrate-phosphate-dextrose and adenine-1 (CPDA-1) anticoagulant. Packed red blood cells were isolated from other blood components by standard blood bank procedures. The red blood cells were mixed with standard lyophilization buffers at a standard blood to buffer ratio. Samples were lyophilized in two separate containers to allow separate measurements of in vitro red cell parameters. One lyophilized sample was sent to the study site at St. Elizabeth's Hospital Blood Transfusion Center in Boston for reconstitution, labeling with ^{51}Cr and subsequent infusion into the autologous donor. The remaining lyophilized sample was retained at CryoPharm and reconstituted on the same day and with the same reconstitution protocol. Each volunteer received 14 mL of packed autologous reconstituted, lyophilized red blood cells through a 20 gauge needle via a scalp vein in the right arm. Peripheral blood samples were taken at selected time points from an indwelling catheter in the contralateral arm. The raw radioactive counts per minute (cpm) were used to calculate both the extrapolated and theoretical time zero (t_0) point following correction for chromium elution and isotopic decay. Urine collections during the first 24 hours post-infusion were also counted to measure the level of isotope clearance via the kidneys. In addition, gamma camera imaging and external probe counts over the heart, spleen and liver at 4 and 24 hours post-infusion were performed to determine the organ distribution profile of the injected dose of reconstituted, lyophilized red blood cells.

Experimental Results From Clinical Study

A. Biochemical and Oxygen Carrying Properties

As part of the design of our clinical study, the levels of important glycolytic intermediates such as , adenosine 5'triphosphate (ATP); 2,3-diphosphoglycerate (2,3-DPG) and lactate were measured on aliquots of each volunteer's blood before and after lyophilization. In addition to glycolytic intermediates, the oxygen carrying capacities of each volunteer's fresh and lyophilized red blood cells were measured using the oxygen tension at which the hemoglobin is 50% saturated (P_{50}) as an index, Tables 2A-2D. The levels of glycolytic intermediates, ATP, 2,3-DPG and lactate in reconstituted lyophilized red blood cells are not significantly different from fresh autologous RBC. Overall glycolysis was assessed by the amount of lactate formed. The oxygen carrying capacities of lyophilized RBC are the same as fresh autologous RBC. All the above data together demonstrate that lyophilized RBC maintained normal physiologic functions upon rehydration.

B. Biophysical Properties and Cell Indices

Important red cell biophysical properties such as red cell deformability and filterability along with standard hematological parameters such mean cell volume (MCV), mean cell

hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) were measured in autologous red blood cells before and after lyophilization. Maintenance of normal biophysical properties are essential if lyophilized RBC are to carry out their normal physiologic function of oxygen transport in vivo. Results in Tables 2A-2D show that reconstituted , lyophilized red blood cells maintained normal cell indices and rheological properties upon rehydration.

C. Post Transfusion 24 hour In Vivo Survival and Circulating Half Life

Reconstituted, lyophilized autologous red blood cells were labeled with isotopic chromium and then infused into autologous donors according to standard procedures outlined in the clinical protocol. The in vivo survival data at different time points for each volunteer are show in Tables 3-6. The raw data are expressed as percent of the injected dose corrected for dilution in the whole body as estimated from body weight and height (reference clinical protocol, section 2). The raw data have also been corrected for chromium elution and isotopic decay. Survival and half life in vivo (T_{50}) are determined using the extrapolated time zero (T_0) as 100% recovery. Validity of the calculation was checked via comparison of extrapolated and theoretical value based on total injected dose and calculation of red cell volume by height and weight measurements, Tables 3-6. The measured mean 24 hour in vivo survival was 89.9$\pm$4.1% of the injected dose for all the four volunteers combined. This level of extended in vivo circulation is comparable to that which has been reported for fresh non-lyophilized red blood cells. In order to calculate the T_{50} of the transfused, lyophilized red blood cells, peripheral blood samples were collected over a maximum of 23 day period. The mean circulating half life of the infused reconstituted, lyophilized RBC from the four volunteers was 25 $\pm$ 5 days. Mean half life for fresh, non-lyophilized red blood cells as measured by ^{51}Cr red cell survival is about 28-30 days corresponding to a mean lifespan of 115-120 days (references 1 and 2). The above in vivo survival data and half life fulfill the requirements by the American Association of Blood Banks and the Food and Drug Administration to qualify a storage method as acceptable.

D. Results from Uptake Probe and Gamma Imaging

Gamma imaging of the three highly vascularized organs of the body was performed to monitor possible sequestration of the transfused red blood cells. In order to monitor the dynamics of organ distribution, measurements were taken at 4 hours and at 24 hours post-infusion. These studies complement measurements of in vivo survival and circulating half life by revealing the mechanism of cell clearance and sequestration. Using gamma camera imaging, the spleen and liver are readily visible from the posterior region while the heart can be more clearly seen from the anterior region. Gamma imaging counts were obtained by direct counting of the radioactivity in the various region and then expressed as ratios and are shown in Table 7 . The distribution of fresh cells in the various organs in standard red cell sequestration studies are approximately the same (i.e., ratio of 1:1:1; heart:liver:spleen). The equal organ distribution of the transfused, lyophilized red blood cells reflects the equilibrium of freely circulating lyophilized cells

among the three highly vascularized organs. In Tables 8A-8C we show the subsequent organ distribution of the infused chromium labeled lyophilized red blood cells, measured using an external uptake probe over marked areas of the body that represented the heart, liver and spleen. The results obtained with an external probe are similar to that with gamma imaging and reflects normal organ distribution of reconstituted, lyophilized red blood cells.

E. Percent of Injected Dose Excreted in Urine

Urine samples were collected for 24 hours post-infusion in order to determine the level of ^{51}Cr cleared by the kidney according to standard procedures for in vivo survival study. Excessive breakdown or lysis of RBC will lead to increased levels of radioactivity in the urine. Note that in any sequestration study, elution of free ^{51}Cr from hemoglobin occurs at a reproducible rate of about 1% per day post transfusion (reference3). Free ^{51}Cr is rapidly filtered by the kidneys and excreted in the urine (reference 4). Abnormal level of hemoglobin resulting from intravascular lysis may lead to hemoglobinuria and saturation of the serum haptoglobin. In Tables 9A-9C we show that the loss of ^{51}Cr in urine is relatively low (4-6% of the injected dose) and well within the acceptable limit for transfused fresh red blood cells. This low level of isotope in urine reflects normal elution of ^{51}Cr as well as possible lysis of the infused RBC.

F. Clinical Observations and Blood Chemistry

There were no significant changes in either the vital signs (body temperature, blood pressure and heart rate) or in the levels of blood components (proteins, haptoglobin, albumin, etc.,) during the initial 24 hours and 7 days post-infusion, Tables 10A-10B . In addition there were no associated clinical side effects due to the infused lyophilized red blood cells. Note also that standard blood bank crossmatch compatibility testing of the reconstituted, lyophilized red blood cells were performed to determine if there was any alteration in red cell surface antigens. There were no observed alterations in surface antigens due to the lyophilization procedure, Tables 10A-10B.

CONCLUSIONS

The data from this clinical study indicate that using the current CryoPharm lyophilization procedure:

1. Lyophilized red blood cells maintained normal biophysical and biochemical properties and normal hematological cell indices upon rehydration .

2. Upon rehydration, lyophilized red blood cells can exhibit normal in vivo survival as measured by the ^{51}Cr single isotope labeling technique.

3. No differential sequestration of the transfused , reconstituted, lyophilized red cells was observed by external monitoring of the heart, liver and spleen with an external uptake probe and gamma imaging.

4. Chromium elution into the urine appeared normal.

5. No changes in vital signs or blood chemistry were associated with the infusion of the low starting dose used in this study.

6. Reconstituted, lyophilized red blood cells maintained normal osmotic deformability profiles. Osmotic deformability profiles of all four subjects are also included in this report (Tables 11A-11D). Note that manitenance of normal deformability profile is important for normal in vivo survival.

It is evident from the above results that significant progress has been made in developing lyophilization procedures that allow lyophilization of human red blood cells such that normal in vivo properties are maintained. Further studies are still required to evaluate safety and efficacy issues that are related to transfusion of large dose of lyophilized red cells.

FUTURE PLANS

Results from the in vivo survival study are very encouraging in that it demonstrates for the first time that small doses of reconstituted, lyophilized red blood can be infused into healthy autologous donors with maintenance of normal physiologic functions . Future studies will need to address the following issues:

1. Scale-up: In the last clinical study, 14mls of lyophilized red blood cells was infused into autologous donors. We will begin to address the issues of safety and toxicity issues relative to infusion of larger volumes of reconstituted lyophilized red blood cells.

2. Cycle development and Container design: Currently, the present lyophilization procedures can accommodate a clinical size sample. A proprietary container is presently being evaluated with the hope that this will provide more efficient lyophilization of human red blood cells.

3. Storage Stability Studies: We have started various experiments some of which are still ongoing to determine the shelf life of our lyophilized red blood cells. The main goal of our research activities in this area is to determine the nature of the biochemical and biophysical mechanisms that are responsible for cell damage during prolonged storage. Understanding the mechanisms which protects RBC from storage induced cell damage will be the key to our developing processing conditions that will extend shelf life.

4. Determination of the relationship between storage stability, residual moisture and in vivo survival. Part of our research efforts will be directed at designing experiments that will allow detailed evaluation of the effects of residual moisture on in vivo survival and oxygen carrying capacity of red blood cells. In vivo survival of red blood cells dried to different levels of residual moisture will be assessed using animal models.

5. Begin detailed analysis of the various components of our buffers with respect to residual amounts in lyophilized samples. Our research activities are directed towards development of analytical procedures that will allow measurement of microquantities of our buffer components.

6. Simplification of the washing procedures. The current rehydration protocol involves two washing steps with dextrose saline. The ultimate goal of our research in this area is to eliminate the need for washing. Experiments will be designed to determine the in vivo survival characteristics of lyophilized red blood cells that have been reconstituted with different wash protocols (using animal models).

7. Begin to gather all in vitro experimental data that are required for filing of an IND at the beginning of 1993.

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TABLE 1: CRYOPHARM CORPORATION RESEARCH MILESTONE CHART-1989

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%	>60%	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	>4 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...

TABLE 2A: AUTOLOGOUS CELL CIRCULATION STUDY: IN VITRO CELL INDICES

IN VITRO CELL INDICES	VOLUNTEER # 92-00001	
	FRESH RBC	LYOPHILIZED
Overall Recovery (%)	NA	87.7
Osmotic Fragility (%)	2.6	2.6
MCV (fl)	87.1	90.4
MCH (pg)	30.7	30.3
MCHC (g/dL)	35.2	33.5
OXYHEMOGLOBIN (%)	96.9	98.5
METHEMOGLOBIN (%)	0.0	0.0
HEMICHROME (%)	3.1	1.5
ATP (μmol/g Hb)	3.57	2.96
2,3-DPG (μmol/g Hb)	10.80	6.51
Lactate ((μmol/mL RBC)	0.86	1.23
P_{50} (mmHg)	27.0	25.5
Deformability Index (DI_{max})	0.604	0.514
Percent of Control DI_{max} (%)	NA	85.1
Relative Filtration Index (RFI)	0.920	0.880
Percent of Control RFI (%)	NA	95.7

Abbreviation: NA, Not Applicable

TABLE 2B: AUTOLOGOUS CELL CIRCULATION STUDY: IN VITRO CELL INDICES

IN VITRO CELL INDICES	VOLUNTEER # 92-00003	
	FRESH RBC	LYOPHILIZED
Cell Recovery (%)	NA	84.5
Osmotic Fragility (%)	1.9	1.2
MCV (fl)	89.4	91.8
MCH (pg)	31.3	30.7
MCHC (g/dL)	35.0	33.4
OXYHEMOGLOBIN (%)	100.0	99.6
METHEMOGLOBIN (%)	0.0	0.0
HEMICHROME (%)	0.0	0.4
ATP (μmol/g Hb)	1.77	1.55
2,3-DPG (μmol/g Hb)	14.40	12.28
Lactate ((μmol/mL RBC)	1.11	0.60
P_{50} (mmHg)	29.5	29.5
Deformability Index (DI_{max})	0.608	0.542
Percent of Control DI_{max} (%)	NA	89.2
Relative Filtration Index (RFI)	1.0	1.0
Percent of Control RFI (%)	NA	100.0

Abbreviation: NA, Not Applicable.
Note: Cell deformability were measured with ektacytometer (reference 5) and gravity driven filtration device (reference 6). Glycolytic intermediates were measured using previously described methods (reference 7)

TABLE 2C: AUTOLOGOUS CELL CIRCULATION STUDY: IN VITRO CELL INDICES

IN VITRO CELL INDICES	VOLUNTEER # 92-00006	
	FRESH RBC	LYOPHILIZED
Cell Recovery (%)	NA	87.1
Osmotic Fragility (%)	4.1	1.5
MCV (fl)	90.7	93.8
MCH (pg)	32.7	30.2
MCHC (g/dL)	36.0	32.2
OXYHEMOGLOBIN (%)	98.9	96.9
METHEMOGLOBIN (%)	0.0	0.0
HEMICHROME (%)	1.1	3.1
ATP (µmol/g Hb)	3.34	3.31
2,3-DPG (µmol/g Hb)	14.14	11.52
Lactate ((µmol/mL RBC)	0.75	0.92
P_{50} (mmHg)	28.0	28.0
Deformability Index (DI_{max})	0.577	0.524
Percent of Control DI_{max} (%)	NA	90.8
Relative Filtration Index (RFI)	1.0	0.920
Percent of Control RFI (%)	NA	92.0

TABLE 2D: AUTOLOGOUS CELL CIRCULATION STUDY: IN VITRO CELL INDICES

IN VITRO CELL INDICES	VOLUNTEER # 92-00007	
	FRESH RBC	LYOPHILIZED
Cell Recovery (%)	NA	81.6
Osmotic Fragility (%)	2.6	3.6
MCV (fl)	85.2	87.7
MCH (pg)	28.2	28.7
MCHC (g/dL)	33.4	32.8
OXYHEMOGLOBIN (%)	100.0	99.3
METHEMOGLOBIN (%)	0.0	0.0
HEMICHROME (%)	0.0	0.7
ATP (µmol/g Hb)	3.57	3.15
2,3-DPG (µmol/g Hb)	12.93	9.02
Lactate ((µmol/mL RBC)	1.14	0.69
P_{50} (mmHg)	27.0	26.0
Deformability Index (DI_{max})	0.573	0.533
Percent of Control DI_{max} (%)	NA	93.0
Relative Filtration Index (RFI)	1.0	1.0
Percent of Control RFI (%)	NA	100.0

Abbreviation: NA, Not Applicable

Table 3: Subject # 1 : half life is 24 days

Data Analysis of In-Vivo Survival of 51Chromium Labeled Reconstituted Red Blood Cells Unit #R9200001

Time Point	cpm	Net Cpm	Hematocrit	Net cpm/ml	Mean Net cpm/ml	% Ext. To	% Theor. To
5 minutes	2550.0/2561.2	2464.3/2475.5	43.0	5902.9/5929.7	5916.3	100.0	98.7
7.5 minutes	2568.4/2589.6	2483.7/2503.9	43.5	5878.6/5928.8	5903.7	99.8	98.5
12 minutes	2534.2/2532.1	2448.5/2446.4	43.0	5865.0/5860.0	5862.5	99.1	97.7
15 minutes	2514.8/2523.5	2429.1/2437.8	43.0	5818.5/5839.4	5829.0	98.6	97.2
20 minutes	2492.6/2507.7	2406.9/2422.0	44.0	5634.3/5669.7	5652.0	95.6	94.3
30 minutes	2485.8/2489.7	2400.1/2403.3	42.5	5816.7/5824.5	5820.6	98.4	97.1
45 minutes	2394.8/2416.3	2309.1/2330.6	42.5	5596.2/5648.3	5622.3	95.1	93.8
60 minutes	2451.7/2443.2	2366.0/2357.5	43.0	5667.4/5647.0	5657.2	95.7	94.3
2 hours	2415.0/2427.8	2329.3/2342.1	43.0	5579.5/5610.1	5594.8	94.6	93.3
4 hours	2325.0/2344.7	2239.3/2259.0	not done	5401.6/5449.1	5425.4	91.8	90.5
6 hours	2313.2/2316.7	2227.5/2231.0	not done	5373.1/5381.6	5377.4	90.9	89.7
24 hours 1	2244.1/2254.0	2158.4/2168.3	not done	5307.5/5331.9	5319.7	90.0	88.7
48 hours 2	2276.2/2240.5	2190.5/2154.8	43.0	5399.8/5311.8	5355.8	90.6	89.3
72 hours 3	2158.0/2155.8	2073.2/2070.1	42.0	5281.7/5273.8	5277.8	89.3	88.0
96 hours 4	2112.1/2108.2	2026.4/2022.5	41.0	5337.8/5327.6	5332.7	90.2	88.9
192 hours 8	1605.1/1662.3	1519.4/1576.6	43.0	3957.5/4106.5	4032.0	68.2	67.2
384 hours 16	1370.8/1358.0	1285.1/1272.3	42.0	3732.9/3695.7	3714.3	62.8	61.9
* 624 hours 22	1117.4/1123.3	2616.9/2630.7	43.0	2598.6/2612.3	2605.5	44.1	43.5

Background cpm 85.7

Standard cpm 952,809

Whole Blood Volume = 5.520

Red Cell Volume = 2.3184L

Theorectical T0 = 5,996.2

Total Injected Dose = 13,901,483.3

Extrapolated T0 = 5,913.0

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Table 4: Subject # 2, half life is 29 days

Data Analysis of In-Vivo Survival of 51Chromium Labeled Reconstituted Red Blood Cells Unit #R9200003

Time Point	cpm	Net Cpm	Hematocrit	Net cpm/ml	Mean Net cpm/ml	% Ext. To	% Theor. To
5 minutes	2845.4/ND	2753.6/ND	43.5	6330.1/ND	6330.1	99.9	100.0
7.5 minutes	2865.2/2849.0	2773.4/2757.2	43.5	6375.6/6338.4	6357.0	100.0	100.0
12 minutes	2853.1/2845.2	2761.3/2753.4	43.5	6347.8/6329.7	6338.8	100.0	100.0
15 minutes	2794.6/2786.2	2702.8/2694.4	42.5	6359.5/6339.8	6349.7	100.0	100.0
20 minutes	2811.3/2808.8	2719.5/2717.0	43.0	6324.4/6318.6	6321.5	99.8	100.0
30 minutes	2764.5/2776.5	2672.7/2684.7	43.5	6144.1/6171.7	6157.9	97.2	100.0
45 minutes	2779.5/2773.0	2687.7/2681.2	44.0	6108.4/6093.6	6101.0	96.3	100.0
60 minutes	2746.1/2709.7	2654.3/2617.9	44.0	6032.5/5949.8	5991.2	94.6	98.7
2 hours	2744.0/2748.3	2652.2/2656.5	43.5	6097.0/6106.9	6102.0	96.3	100.0
4 hours	2627.7/2623.0	2535.9/2531.2	41.5	6110.6/6099.3	6105.0	96.4	100.0
6 hours	2535.1/2532.3	2443.3/2440.5	42.0	5817.4/5810.7	5814.1	91.8	95.7
24 hours 1	2606.1/2620.9	2514.3/2539.1	42.0	5986.4/6021.7	6004.0	94.8	98.9
48 hours 2	2367.6/2375.5	2275.8/2283.7	40.0	5689.5/5709.3	5699.4	90.0	93.9
72 hours 3	2282.6/2250.9	2190.8/2159.1	38.0	5765.3/5681.8	5723.6	90.4	94.3
96 hours 4	2180.8/2185.2	2089.0/2093.4	40.0	5222.5/5233.5	5228.0	82.5	86.1
216 hours 9	1957.5/1959.3	1865.7/1867.5	39.0	4783.8/4788.5	4786.2	75.6	78.8
360 hours 15	1734.1/1751.9	1642.3/1660.1	39.0	4211.0/4256.7	4233.9	66.8	69.7
528 hours 22	1203.1/1209.0	1448.3/1492.0	40.0	3620.8/3730.0	3675.4	58.0	60.5

Background cpm 91.8
Whole Blood Volume = 5.880

Red Cell Volume = 2.587L
Theoretical T0 = 6,072.3

Standard cpm 1,556,888.0
Total Injected Dose = 15,708,999.9
Extrapolated T0 = 6,334.8

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Table 3: Subject # 3, half life is 27 days

Data Analysis of In-Vivo Survival of 51Chromium Labeled Reconstituted Red Blood Cells Unit #R9200006

Time Point	cpm	Net Cpm	Hematocrit	Net cpm/ml	Mean Net cpm/ml	% Ext. To	% Theor. To
5 minutes	2454.2/2441.0	2366.9/2353.7	43.0	5504.4/5473.7	5489.1	97.9	90.0
7.5 minutes	2420.1/2404.2	2332.8/2316.9	43.5	5362.8/5326.2	5344.5	95.3	87.6
12 minutes	2323.4/2297.1	2236.1/2209.8	43.5	5140.5/5080.0	5110.3	91.2	83.8
15 minutes	2335.4/2309.8	2248.1/2222.5	43.0	5228.1/5168.6	5198.4	92.7	85.2
20 minutes	2227.8/2243.9	2140.5/2156.6	42.5	5036.5/5074.4	5055.5	90.2	82.9
30 minutes	2228.8/2233.5	2141.5/2146.2	42.5	5038.8/5049.9	5044.4	90.0	82.7
45 minutes	2178.6/2188.2	2091.3/2100.9	41.5	5039.3/5062.4	5050.9	90.1	82.8
60 minutes	2223.8/2211.7	2136.5/2124.4	42.0	5086.9/5058.1	5072.5	90.5	83.1
2 hours	2321.6/2319.0	2234.3/2231.7	43.0	5196.0/5190.0	5193.0	92.6	85.1
4 hours	2156.8/2191.9	2069.5/2104.6	41.5	4986.7/5071.3	5029.0	89.7	82.4
6 hours	2143.4/2145.8	2056.1/2058.5	41.0	5014.9/5020.7	5017.8	89.5	82.3
24 hours 1	2209.5/2190.9	2122.2/2103.6	44.5	4769.0/4727.2	4748.1	84.7	77.8
48 hours 2	2068.3/2042.8	1981.0/1955.5	43.0	4607.0/4547.7	4577.4	81.7	75.0
72 hours 3	2039.9/2042.7	1952.6/1955.4	44.0	4437.7/4444.0	44440.9	79.2	72.8
96 hours 4	1924.1/1964.3	1836.8/1877.0	43.0	4271.6/4365.1	4318.4	77.0	70.8
192 hours 8	1877.3	1877.3	45.0	4171.8	4171.8	74.4	68.4
360 hours 15	1423.6	1423.6	45.0	3163.6	3163.6	56.4	51.9
552 hours 23	1203.6	1203.6	44.0	2735.4	2735.4	48.8	44.8

Background cpm 87.3 Standard cpm 1,329,441.0

Whole Blood Volume = 5.371 Red Cell Volume = 2.310L Total Injected Dose = 14,092,074.6

Theorectical T0 = 6,100.5 Extrapolated T0 = 5,605.9

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Table 6: Subject # 4, half life is 20 days

Data Analysis of In-Vivo Survival of 51Chromium Labeled Reconstituted Red Blood Cells Unit #R9200007

Time Point	cpm	Net Cpm	Hematocrit	Net cpm/ml	Mean Net cpm/ml	% Ext. To	% Theor. To
5 minutes	3076.6	2987.9	39.0	7661	7661	100.6	108.5
7.5 minutes	3094.6	3005.9	40.0	7515	7515	98.6	106.5
12 minutes	3066.5	2977.8	39.0	7635	7635	100.2	108.1
15 minutes	3044.7	2956	39.0	7579	7579	99.5	107.4
20 minutes	2947.1	2858.4	39.0	7329	7329	96.2	103.8
30 minutes	2912.0	2823.3	34.0	8304	8304	109.0	117.6
45 minutes	2894.7	2806.0	38.0	7384	7384	96.9	104.6
60 minutes	2809.5	2720.8	38.0	7161	7161	94.0	101.4
2 hours	3010.7	2922	40.0	7305	7305	95.9	103.5
4 hours	2779.9	2691.2	39.0	6901	6901	90.6	97.8
6 hours	2792.1	2703.4	39.0	6932	6932	91.0	98.2
24 hours 1	2769.5	2680.8	39.0	6874	6874	90.2	98.2
48 hours 2	2647.6	2558.9	40.0	6397	6397	84.0	90.6
72 hours 3	* NOT DONE	ND	ND	ND	ND	ND	ND
96 hours 4	* NOT DONE	ND	ND	ND	ND	ND	ND
192 hours 8	* NOT DONE	ND	ND	ND	ND	ND	ND
408 hours 17	1620.6	1620.6	40.0	4051.5	4051.5	53.2	57.4
528 hours 22	1301.4	1301.4	40.0	3253.5	3253.5	42.7	46.1

Background cpm 88.7

* SUBJECT UNAVAILABLE

Standard cpm 1,329,441.0

Whole Blood Volume = 5.89

Red Cell Volume = 2.356L
Theoretical T0 = 7059.8

Total Injected Dose = 16,632,911
Extrapolated T0 = 7618.6

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Table 7: GAMMA IMAGING RESULTS

	Spleen/Heart	Liver/Heart	Spleen/Liver
Subject #1			
4 Hours	0.96	0.67	1.43
24 Hours	1.16	0.73	1.58
Subject # 2			
4 Hours	0.71	0.66	1.08
24 Hours	0.80	0.72	1.11
Subject # 3			
4 Hours	1.20	0.65	1.86
24 Hours	1.10	0.55	2.00
Subject # 4			
4 Hours	0.99	0.65	1.86
24 Hours	1.04	0.53	1.95

TABLES 8A-8C: MEASUREMENT OF DISTRIBUTION OF RADIOLABELLED RECONSTITUTED, LYOPHILIZED RBC USING EXTERNAL UPTAKE PROBE

TABLE 8A: SUBJECT # 1

TIME POINTS	HEART	LIVER	SPLEEN	SPLEEN/ HEART	LIVER/ HEART	SPLEEN/ LIVER
4 Hours	28,250	19,133	27,305	0.957	0.671	1.427
24 Hours	25,022	18,341	29,042	1.161	0.733	1.583

TABLE 8B: SUBJECT # 2

TIME POINTS	HEART	LIVER	SPLEEN	SPLEEN/ HEART	LIVER/ HEART	SPLEEN/ LIVER
4 Hours	37,789	24,873	26,864	0.711	0.658	1.080
24 Hours	33,091	23,786	24,477	0.800	0.719	1.110

TABLE 8C: SUBJECT # 3

TIME POINTS	HEART	LIVER	SPLEEN	SPLEEN/ HEART	LIVER/ HEART	SPLEEN/ LIVER
4 Hours	35,848	23,207	43,164	1.204	0.647	1.860
24 Hours	34,048	18,686	37,602	1.104	0.549	2.0

TABLES 9A-9C: LEVELS OF RADIOACTIVITY EXCRETED IN URINE FROM HUMAN VOLUNTEERS

TABLE 9A: SUBJECT # 1

TIME POINTS	VOLUME OF URINE (mL)	CPM	MEAN CPM/mL	TOTAL CPM
0 - 6 Hours	300	877.0	781.5	234,450
6 - 12 Hours	200	1,165.0	1,069.5	213,900
12 - 18 Hours	0	0	0	0
18 - 24 Hours	470	573.0	477.5	224,425
% Injected Dose Excreted in Urine	-		-	4.1

TABLE 9B: SUBJECT # 2

TIME POINTS	VOLUME OF URINE (mL)	CPM	MEAN CPM/mL	TOTAL CPM
0 - 6 Hours	400	788.4	698.7	279,480
6 - 12 Hours	370	852.2	762.5	282,125
12 - 18 Hours	240	711.3	621.6	149,184
18 - 24 Hours	190	873.8	784.1	148,979
% Injected Dose Excreted in Urine	-	-	-	5.5

TABLE 9C: SUBJECT # 3

TIME POINTS	VOLUME OF URINE (mL)	CPM	MEAN CPM/mL	CPM/mL
0 - 6 Hours	780	555.0	473.5	369,330
6 - 12 Hours	610	436.0	354.5	216,245
12 - 18 Hours	945	160.0	78.5	74,183
18 - 24 Hours	1,290	264.0	182.5	235,425
% Injected Dose Excreted in Urine	-	-	-	5.8

** Total counts have been corrected for background.

NOTE: % Injected Dose Excreted in Urine = $\frac{\text{Total Overall Urine CPM x 100}}{\text{Injected Dose}}$

TABLE 10A: CLINICAL OBSERVATIONS AND BLOOD CHEMISTRY

ASSAYS	SUBJECT #1		SUBJECT # 2		NORMAL RANGE
	PRE	POST	PRE	POST	
Protein (g/dL)	7.0	6.60	7.40	6.90	6.0 - 8.0
ALB (g/dL)	4.30	4.10	4.60	4.30	3.50 -5.0
T BIL (mg/dL)	0.60	0.40	0.60	0.70	0.0 - 1.5
ALK P (IU/L)	82.0	76.0	54.0	51.0	43 -122
SGPT (IU/L)	42.0	39.0	46.0	47.0	7 - 56.0
SGOT (IU/L)	17.0	24.0	28.0	26.0	5 - 40
LDH (IU/L)	408	518	317	366	313 - 618
Haptoglobin (md/dL)	ND	58	ND	<5.0	27 -139
*Vital Signs	Norm	Norm	Norm	Norm	Norm
Crossmatch	Comp	Comp	Comp	Comp	Comp

Abbreviations: ALB, Albumin; T BIL, Total Bilirubin; ALK P, Alkaline Phosphatase Isoenzymes; SGPT, Serum Alanine Aminotransferase; SGOT, Serum Aspartate Aminotransferase; LDH, Lactate Dehydrogenase; Norm, Normal; Comp, Compatible.

* Includes Temperature, Respiration, Pulse and blood pressure during the initial 24 hours post-infusion. Final check on vital signs was made on Day 7 post-infusion .

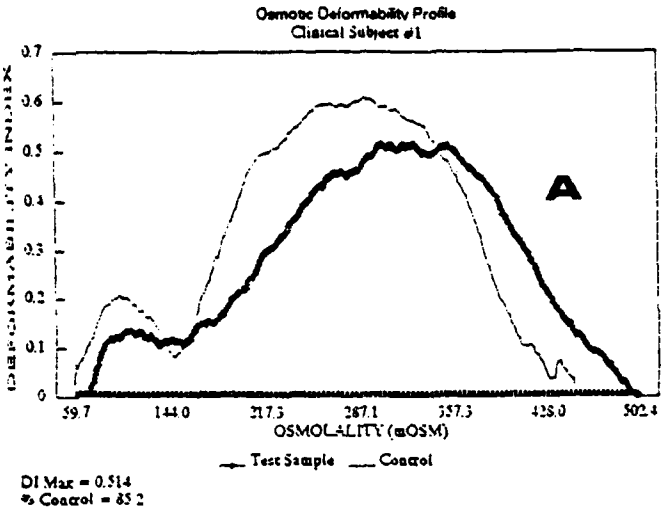
TABLE 10B: CLINICAL OBSERVATIONS AND BLOOD CHEMISTRY

ASSAYS	SUBJECT #3		SUBJECT # 4		NORMAL RANGE
	PRE	POST	PRE	POST	
Protein (g/dL)	6.9	6.50	7.50	7.30	6.0 - 8.0
ALB (g/dL)	4.50	4.20	4.40	4.40	3.50 -5.0
T BIL (mg/dL)	0.60	0.60	0.50	0.40	0.0 - 1.5
ALK P (IU/L)	44.0	44.0	94.0	89.0	43 -122
SGPT (IU/L)	31.0	31.0	43.0	41.0	7 - 56.0
SGOT (IU/L)	32.0	32.0	48.0	41.0	5 - 40
LDH (IU/L)	324	346	416	435	313 - 618
Haptoglobin (md/dL)	45.0	44.0	95.7	87.7	27 -139
*Vital Signs	Norm	Norm	Norm	Norm	Norm
Crossmatch	Comp	Comp	Comp	Comp	Comp

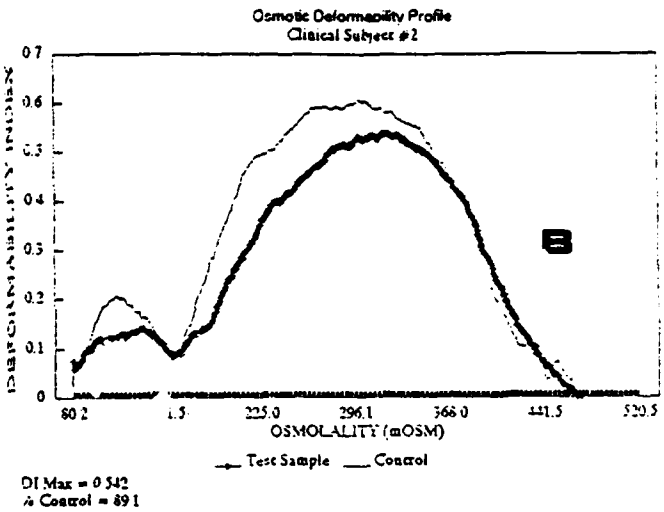
Abbreviations: ALB, Albumin; T BIL, Total Bilirubin; ALK P, Alkaline Phosphatase Isoenzymes; SGPT, Serum Alanine Aminotransferase; SGOT, Serum Aspartate Aminotransferase; LDH, Lactate Dehydrogenase; Norm, Normal; Comp, Compatible.

* Includes Temperature, Respiration, Pulse and blood pressure during the initial 24 hours post-infusion. Final check on vital signs was made on Day 7 post-infusion .

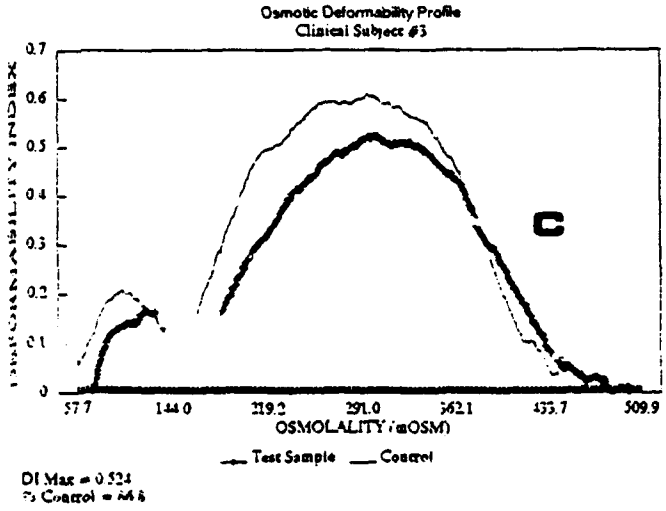
Table 11: Osmotic deformability profiles of fresh red blood cells and reconstituted, lyophilized cells. Note that reconstituted, lyophilized red blood cells have normal osmotic deformability profiles. Maintenance of normal deformability profiles are required for normal physiologic functions.



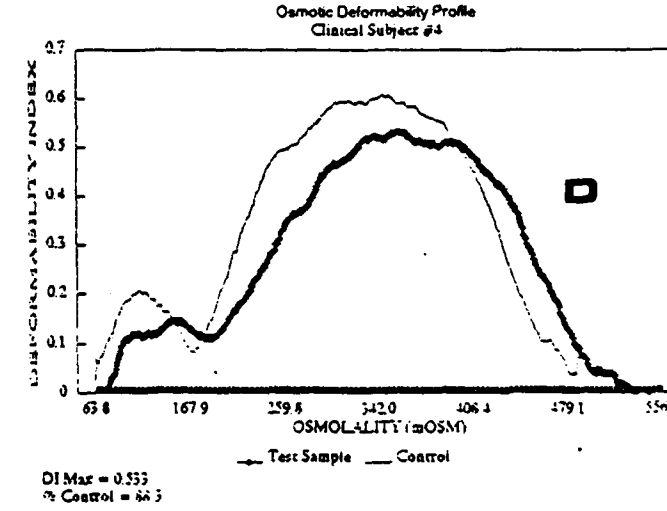
DI Max = 0.514
% Control = 85.2



DI Max = 0.542
% Control = 89.1



DI Max = 0.524
% Control = 86.6



DI Max = 0.553
% Control = 86.3

CRYOPHARM CORPORATION

PROTOCOL

A CLINICAL EVALUATION OF THE SURVIVAL OF AUTOLOGOUS LYOPHILIZED AND RECONSTITUTED 51-CHROMIUM LABELED HUMAN RED BLOOD CELLS IN VIVO

April 1991

TABLE OF CONTENTS

1.0 INTRODUCTION

Cryopharm Corporation has developed a novel freeze-drying process for lyophilizing and reconstituting human red blood cells. The company has evidence that closely related primate red blood cells (macaque or baboon) will not serve as adequate models for making useful improvements in this process, and is therefore conducting this study of very small doses in humans so that further process improvements can be investigated.

Cryopharm has conducted several "dry runs" in which blood was collected, shipped, processed, shipped again, reconstituted, and labeled with 51-Cr. These dry runs were successfully conducted to confirm the logistics and sterility of the procedures used in this study.

2.0 OBJECTIVES

The objective of this study is to determine the in vivo survival of 51-Cr labeled lyophilized and reconstituted human red blood cells.

3.0 STUDY DESIGN AND DESCRIPTION

This single-center study will test the in vivo survival of lyophilized, reconstituted red blood cells in six healthy male volunteers. All transfusions will be performed with autologous blood samples and the study will proceed one subject at a time to minimize cross contamination risks. Eligible study subjects will be phlebotomized one unit (450 ml) of whole blood at the blood donor center of the study site. This blood will be shipped to Cryopharm where it will be washed and the packed red cells will then be lyophilized, and then shipped back to the study site. The lyophilized red cells will be stored refrigerated until use. Fourteen days after phlebotomy, the subject will return to the study site, at which time a 25 ml aliquot of reconstituted 51-Cr labeled autologous red blood cells will be infused intravenously into a large arm vein. The subject will remain in confinement at the study site for 24 hours following the transfusion and return on the following four days (Study Days 2 through 5) to have peripheral blood samples collected for radioactivity counting. The subject will return seven days post-infusion (Study Day 8) for follow-up examination and procedures.

4.0 SUBJECT SELECTION

To be eligible for participation in the study, subjects must meet the following criteria prior to having the blood sample for lyophilization taken:

1. Male, age 21 to 35 years, inclusive.

2. Medical history, vital signs, physical examination including fundoscopic and neurologic examinations, and laboratory tests (Section 5.1.7) without evidence of clinically significant medical condition, in particular, no evidence of hepatitis.

3. A 12-lead electrocardiogram (ECG) without clinically significant abnormality.

4. Weight within the following limits for height:

Height Without Shoes (cm)	Weight Indoor Clothing Without Shoes (kg)
156-158	51-77
159-161	52-97
162-163	54-81
164-166	55-83
167-168	56-85
169-170	57-88
171-173	58-90
174-176	59-93
177-178	60-95
179-181	61-98
182-184	62-100
185-186	63-103
187-189	64-106
190-191	65-109
192-193	67-111

5. No known drug hypersensitivity, atopy or known seasonal or other allergy.

6. Subject does not take any medication on a chronic basis and has not taken any medication including over the counter medication and alcohol within the previous week. In addition, subject has not received any medication with known liver or kidney toxicity in the previous six months.

7. Subject does not have a history of:

   - Renal disorders or BUN, creatinine, uric acid, sodium, potassium or chloride values outside the investigator's normal range at any pretransfusion evaluation.

   - Hepatic disorder or AST, ALT, GGT, LDH, total bilirubin or direct bilirubin values outside the investigator's normal range at any pretransfusion evaluation.

- Bleeding/coagulation disorder or severe anemia.

8. Negative urine drug and blood alcohol screens.

9. Subject is HIV antibody negative.

10. Subject has voluntarily signed the St. Elizabeth's Hospital of Boston Informed Consent Form after the nature of the study has been explained. Subject has also reviewed Appendix I of this protocol.

5.0 STUDY PROCEDURES

5.1 SCREENING PROCEDURES

Seven days prior to phlebotomy (Study Day -21), the following will be obtained to assess compliance with the subject selection criteria outlined in Section 4.0:

1. Informed consent documented in writing.

2. Medical history.

3. Height and weight.

4. Vital signs: sitting blood pressure and pulse, respiratory rate, and temperature.

5. Complete physical examination.

6. 12-lead electrocardiogram.

7. Laboratory Tests: All blood and urine samples will be collected and handled in accordance with accepted laboratory procedures. Blood samples for determination of prothrombin time and activated partial thromboplastin time will be collected after the samples for hematology, and blood chemistry.

   Obtain blood and urine samples after at least an eight-hour fast for the following laboratory tests:

   Hematology: hemoglobin, hematocrit, red blood cell count, white blood cell count with differential, and platelet count.

Blood Chemistry: blood urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST/SGOT), alanine aminotransferase (ALT/SGPT), gamma glutamyl transferase (GGT), lactate dehydrogenase (LDH), alkaline phosphatase, total bilirubin, direct bilirubin, total protein, albumin, uric acid, calcium, inorganic phosphorus, glucose, sodium, potassium, chloride, bicarbonate.

Coagulation: prothrombin time (subject and control) and activated partial thromboplastin time (subject and control).

Urinalysis: pH, specific gravity, albumin, blood, glucose, and microscopic examination to include white blood cells, red blood cells, bacteria, casts, and crystals.

5.2 DAY OF PHLEBOTOMY (STUDY DAY -12)

The following will be obtained twelve days prior to transfusion with radioactively labeled RBCs:

1. Medical history update.

2. Vital signs, as described in Section 5.1.4.

3. Hematocrit, blood alcohol screen, and urine drug screen.

4. Blood collection: One unit of whole blood (450 ml) will be collected by a trained phlebotomist in a standard blood collection bag containing CPD. Subjects will be screened by the phlebotomist according to standard screening procedures for voluntary blood donation. The whole blood then will be shipped at 4°C to Cryopharm (next day delivery). All blood products collected at this time will be subjected to the usual screening procedures (ALT, HIV HB_sA_g) for voluntarily donated blood products.

   Each sample will be processed by Cryopharm (as described in Appendix II) and shipped back to the study site within ten days. Upon arrival, the sealed bag should be kept refrigerated prior to reconstitution.

5.3 MORNING OF TRANSFUSION (STUDY DAY 1)

Subjects will report to the study site on the morning of transfusion when the following will be performed to verify compliance with the subject selection criteria. All results must be available before transfusion.

1. Medical history update.

2. Weight: Each subject will be weighed just before transfusion of labeled cells so that total blood volume can be estimated.

3. Vital signs, as described in Section 5.1.4.

4. Physical examination.

5. Laboratory tests, as described in Section 5.1.7. Be sure to obtain the subject's hematocrit at this time.

6. Urine drug and blood alcohol screen.

5.4 TRANSFUSION PROTOCOL

Following screening procedures, subjects will be admitted to the clinical research unit of the study site as inpatients. They will remain as inpatients for approximately 24 hours following transfusion of autologous blood.

5.4.1 Reconstitution of the RBCs: Lyophilized RBCs from the study subject will be reconstituted at the study site using a Cobe cell washer according to the Cryopharm protocol (refer to Appendix III). Two aliquots (3-5 ml each) of the reconstituted red blood cells will be used to inoculate blood culture bottles. A sample will also be sent to the blood bank for type and crossmatch.

5.4.2 Chromium Labeling of Red Blood Cells: The re-hydrated blood will be brought to the Nuclear Medicine Department where a 30-35 ml aliquot will be removed from the Cobe bag using sterile techniques. These red cells will be incubated for 15 minutes at 37°C with 150-200 microcuries of sterile 51-Cr by standard technique. After quenching with sterile ascorbic acid, the red cells are ready for transfusion. A detailed labeling protocol is included in Appendix III.

5.4.3 Transfusion of Labeled Red Blood Cells: An indwelling catheter with a heparin lock will be inserted into a large vein in the subject's forearm. The labeled autologous red blood cells will be infused through a scalp vein needle into the contralateral arm.

5.4.4 Vital Signs will be measured hourly following transfusion for four hours, then once per shift until hospital discharge, as described in Section 5.1.4.

5.4.5 Blood Sampling for Red Blood Cells Survival: Following administration of labeled cells (t = O), peripheral blood samples (5 ml each) will be collected. Five ml samples of whole blood will be collected into purple stopper (EDTA) blood collection tubes through the indwelling heparin lock. An initial portion will be withdrawn and discarded. The indwelling catheter will be flushed with normal saline following each blood collection.

The whole blood aliquots will be collected at the following times post-transfusion:

	Post-transfusion Blood Collection Times				
Time	Day 1	Day 2	Day 3	Day 4	Day 5
0		X	X	X	X
5 minutes	X				
7.5 minutes	X				
12 minutes	X				
15 minutes	X				
20 minutes	X				
30 minutes	X				
45 minutes	X				
1 hour	X				
2 hours	X				
4 hours	X				
6 hours	X				

The collection of blood will take precedence over all other study activities. Numerous samples are needed in the first hour and several during Day 1 as damaged red cells are very rapidly cleared from circulation. Provided enough radioactivity remains in circulation, the study will collect samples through Day 5. The time that each blood sample is drawn will be recorded to the nearest minute on the appropriate case report form for each subject.

Each 5 mL sample of whole peripheral blood collected in EDTA will be counted in the Nuclear Medicine Department according to their standard protocol (see Appendix III).

5.4.6 Urine Collection: Subjects will be instructed to void and empty their bladders completely just prior to transfusion. Following transfusion, subjects will void into a bedside urinal. Urine volume will be recorded and urine will be pooled into a large plastic bottle at six hourly intervals for gamma counting. The percentage of injected dose excreted in urine will be calculated.

5.4.7 Gamma Imaging and External Counts: Subjects will be taken to nuclear medicine for gamma camera imaging and external counts 4-hours and 24-hours after transfusion of labeled red blood cells. Gamma imaging will be taken using posterior and anterior views. External counts will be taken over the precordium, liver and spleen with the following ratios determined:

liver/precordium
spleen/precordium
spleen/liver

5.5 FOLLOW-UP EVALUATIONS (STUDY DAY 8)

The following procedures and evaluations will be performed seven days post-transfusion.

1. Medical history update.

2. Vital signs, as described in Section 5.1.4.

3. Physical examination.

4. Hematology and blood chemistry laboratory tests, as described in Section 5.1.7.

6.0 MANAGEMENT OF INTERCURRENT EVENTS

6.1 DIETARY RESTRICTIONS

Subjects will be instructed not to consume any alcohol-containing beverage during the period from seven days prior to phlebotomy to the completion of the follow-up evaluations.

6.2 CONCURRENT MEDICATION

No medication, including over-the-counter medication, is to be administered to or taken by any subject from two weeks prior to autologous blood transfusion until after study completion. If a subject reports taking any medication, a medical monitor at Cryopharm must be notified immediately and the incident must be documented on the appropriate case report form.

6.3 ACTIVITY RESTRICTIONS

No vigorous exercise will be permitted from Day -14 until after study completion.

6.4 ADVERSE EVENTS

Throughout the duration of the study and especially for the first 12 hours, the investigator will closely monitor each subject for evidence of transfusion intolerance and for the development of clinical or laboratory evidence of an adverse event or any signs of hypersensitivity reaction. All adverse events which occur during the course of the study must be reported in detail on the appropriate case report form and followed to a satisfactory resolution.

The description of the adverse event will include the date, time of onset, duration, severity, etiology, the relationship of the adverse event to the Investigational lyophilized reconstituted red cells and any treatment required.

If a serious or unexpected adverse event occurs, and/or if a subject dies from any cause during or within one month after investigational red cell administration, whether related to the study or not, one of the following monitors must be notified within 24 hours by telephone:

Christine Williams	Roger Hackett
Cryopharm Corp.	Cryopharm Corp.
(818) 793-1040	(818) 793-1040

A written confirmation of the serious or unexpected adverse event or death, including autopsy report, if available, will be sent to a Cryopharm monitor within five days of the telephone report.

The investigator will rate the severity of any adverse event according to the following definitions:

Mild: The adverse event is transient and easily tolerated by the subject.

Moderate: The adverse event causes the subject discomfort and interrupts the subject's usual activities.

Severe: The adverse event causes considerable interference with the subject's usual activities, and may be incapacitating or life-threatening.

The investigator will use the following definitions to assess the relationship of the adverse event to the investigational red cells:

Probable: The adverse event has a timely relationship to transfusion and a potential alternative etiology is not apparent.

Possible: The adverse event has a timely relationship to transfusion. However, a potential alternative etiology exists which may be responsible for the adverse event.

No relationship: Definite evidence exists that the adverse event is related to an etiology other than transfusion. The etiology must be stated on the case report form.

6.5 PREMATURE DISCONTINUATIONS

Each subject has the right to withdraw from the study at any time without prejudice. The investigator may discontinue any subject's participation when he feels it is necessary for any reason, including adverse event or failure to comply with the protocol.

Should a subject withdraw from the study, the reason(s) must be stated in the case report, and the following evaluations of the subject should be performed: medical history update, vital signs, complete physical examination, opthalmologic examination, neurologic assessment, ECG, EEG, laboratory tests (hematology, coagulation, blood chemistry, and urinalysis), and collection of archive samples.

6.6 MODIFICATION OF PROTOCOL

Neither the investigator nor the Cryopharm monitor will modify this protocol without first obtaining concurrence of the other. The modification must be documented in writing. Any change in the research activity, except that necessary to remove an apparent immediate hazard to the subject, must be reviewed and approved by the St. Elizabeth's Hospital Institutional Review Board prior to implementation. Cryopharm may submit protocol amendments which may be subject to the St. Elizabeth's Hospital Institutional Review Board approval.

6.7 DEPARTURE OF PROTOCOL FOR AN INDIVIDUAL SUBJECT

When a significant departure from the protocol is deemed necessary for an individual subject, the investigator or other physician in attendance must contact the Cryopharm monitor.

Such contact with the Cryopharm monitor will be made as soon as possible to permit a decision as to whether or not the subject is to continue in the study. Any departure from the protocol will be authorized only for that one subject. A description of the departure from the protocol and the reason(s) for it will be recorded on the appropriate case report form.

7.0 CASE REPORT FORMS

Case report forms are provided for each subject. Each form must be filled out completely and legibly in black ink. Corrections of data on the case report form must only be made by crossing out the incorrect values and writing the correct values next to those crossed out. Each correction must be initialed by the investigator or an authorized assistant.

8.0 INSTITUTIONAL REVIEW

Approval must be obtained from the St. Elizabeth's Hospital of Boston Institutional Review Board (IRB) prior to participation of human subjects in these research studies. Confirmation of protocol and informed consent approval and a list of members of the Review Board and their qualifications and affiliations will be provided to Cryopharm prior to the onset of the study.

9.0 SUBJECT CONFIDENTIALITY

All reports and communications relating to subjects in the study will identify each subject only by the subject's initials and by the subject's study number. The investigator agrees to furnish Cryopharm with complete subject identification on the confidential follow-up form, which will be used for purposes of long-term follow-up if needed. This will be treated with strict adherence to professional standards of confidentiality, and will be filed at Cryopharm under adequate security.

10.0 USE OF INFORMATION AND PUBLICATION

All information concerning the blood lyophilization process and Cryopharm operations, such as Cryopharm's patent applications, formulas, manufacturing processes, basic scientific data, or formulation information, supplied by Cryopharm and not previously published is considered confidential information.

The information developed during the conduct of this clinical study is also considered confidential and will be used by Cryopharm in connection with the development of the red blood cell lyophilization process. This information may be disclosed as deemed necessary by Cryopharm. To allow for the use of the information derived from this clinical study and to insure complete and thorough analysis, the investigator is obligated to provide Cryopharm with complete test results and all data developed in this study.

This confidential information shall remain the sole property of Cryopharm, shall not be disclosed to others without the written consent of Cryopharm, and shall not be used except in the performance of this study.

Should the investigator choose to publish the results of this study, a copy of the manuscript will be provided to Cryopharm at least 40 days prior to the date of submission to the intended publisher. In the event Cryopharm chooses to publish the data from this study, a copy will be provided to the investigator at least 40 days prior to the date of submission to the intended publisher.

11.0 INVESTIGATOR'S AGREEMENT

I have received and reviewed the Executive Summary, "Information for Clinical Investigator", for Cryopharm's red blood cell lyophilization process.

I have read the protocol and agree to:

A. Conduct the study as outlined herein

and

B. Maintain the confidentiality of all information received or developed in connection with this protocol.

_____ / /

Signature of Principal Investigator — Date

TABLE I

STUDY SCHEMATIC

INFORMED CONSENT

NOTE: Appendix I to Protocol

1. I understand that my eligibility to fully participate in the study will be determined, in part, by a medical history, physical examination, electrocardiogram, blood chemistry and hematology tests, and urine tests. The screening of these blood tests will require approximately two tablespoons of my blood. They will include a complete blood count, kidney and liver function tests, and tests to determine whether I have been exposed to hepatitis B virus and to HIV (AIDS).

2. I understand that if I remain eligible to participate in the study following screening, a unit (approximately 1 pint) of my blood will be withdrawn through a large vein in my arm. This process will be performed according to the procedures for voluntary blood donation at St. Elizabeth's Hospital. I understand that this procedure will include having my blood pressure and pulse rate determined, having my hematocrit (similar to blood count) determined, and filling out a questionnaire regarding my risk of exposure to the HIV (AIDS) virus. A brief medical history will also be obtained by the phlebotomist (person who draws my blood). At this point I understand that, although I am participating in a research project, this blood donation must be taken seriously. I will refrain from vigorous exercise once I have given the pint of blood and until my follow-up examination is completed. I understand that a sample of the blood collected from me will be screened for exposure to hepatitis or HIV according to standard blood donor procedures.

3. On the morning of transfusion of my re-hydrated freeze-dried red blood cells, I will report to the study site for an interim medical history and physical examination. Blood samples will be obtained (two tablespoons) for hematology and chemistry tests and a blood alcohol level. I will also provide a fresh urine specimen for drug screening. A small catheter will be inserted into a vein in my arm for the purpose of obtaining blood samples during the 24 hours. About two tablespoons of my freeze-dried and rehydrated blood cells will be made radioactive with ^{51} chromium and infused into my other arm through a hypodermic needle. No more than 200 microcuries of radioactive label will be used. This amounts to 2.4 millirads of radiation exposure, which is less than 40% of the amount of radiation from a chest x-ray. Blood samples (1 teaspoon each) will be obtained through the catheter in my arm 8 times during the first hour after transfusion, 2 hours after transfusion, 4 hours after transfusion and 6 hours after transfusion. This will be to determine how long the radioactive blood cells can be detected in my veins. I will be admitted to the hospital and remain in the hospital for 24 hours after the transfusion. During this time I will collect all of my urine in a plastic container as instructed by my nurse. Twenty four hours after the transfusion, another blood sample (1 teaspoon) will be obtained, the catheter will be removed from my arm, and I will be discharged from the hospital.

4. I will return to the study site daily for the following four days to have a blood sample (1 teaspoon each) withdrawn from an arm vein.

5. I will return to the study site for a follow-up history, physical examination and laboratory evaluation seven days after transfusion. At this time up to two tablespoons of blood will be drawn for blood chemistry and hematology testing.

6. I understand that I am not to consume any alcoholic beverage during the period from screening evaluation (19 days prior to transfusion) through completion of the follow-up evaluation (the 7th day following transfusion). In addition, I understand that I must not consume medication, including non-prescription medication, during this time. If I do take medication during the study I must promptly notify Dr. Weinstein at (617) 789-3000, X3081.

7. I understand that the procedures performed for the purpose of the study are being performed for clinical research, and will be performed at no cost to me. I will be compensated at the rate of $50.00 per day for each active day of participation in the study, including screening evaluation, phlebotomy, five days of blood sampling after the transfusion, and follow-up visit. A bonus of $100.00 will be paid for successfully completing the entire study. The total potential remuneration to me for participating in the study is $500.00.

8. I understand that the cell circulation results obtained during the initial few hours following the injection of my radioactive red blood cells may not warrant continuation of the study. In this case I will remain in the hospital for the first 24 hours after injection of the radioactive cells, but I may not have to return on the following four days to provide a blood sample. I will return to the hospital for the scheduled follow-up examination. I understand that if the study is shortened in this manner, my potential total remuneration will remain $500.00 provided I successfully complete the study through follow-up.

_____ _____

Signature of Volunteer Date

_____ _____

Clinical Coordinator Date

APPENDIX II

The Lyophilization of Red Blood Cells at Cryopharm

Each unit of whole blood received from the St. Elizabeth's clinical coordinator will be inspected and released by Quality Control at Cryopharm prior to processing in the Company's certified class 100 clean room (A class 100 rating denotes fewer than 100 particles per cubic foot that are 0.5 micron or larger). The clean room is routinely disinfected and tested for viable microbials. The whole blood will be processed using standard procedures to produce packed red blood cells. The packed cells will be washed using a Cobe Cell washer and sterile dextrose saline (commercially available). The washed cells and the sterile lyophilization buffer will be aseptically transferred into a pre-weighed lyophilization bag. Cryopharm's lyophilization blood bags are manufactured from approved blood bag materials and are certified as sterile by the manufacturer (Ethox Corporation of Buffalo, NY is a manufacturer of standard blood bags). Cryopharm personnel will record the weight of the "Processed Lyophilization Bag", and load the bag into the sterile lyophilizer chamber when the shelf temperature has equilibrated at -20°C. The cells will then be frozen in place and lyophilized according to the current standard operating procedures for the lyophilization of human red blood cells. The time and temperature of the drying cycle will depend on the current Cryopharm technology. The current drying procedure will require approximately 170 hours. At the end of the cycle, sterile ultrapure nitrogen will be used to purge the lyophilizer chamber to keep the moisture away from the dried cells before the bag is sealed. The sealed bag will be stored at 4°C, and placed under Q.C. quarantine. Q.C. personnel will inspect the quality of the dried cells, and will determine the percent weight loss of each dry unit. Only the unit that has met all product specifications will be shipped to the study site for reconstitution and transfusion.

APPENDIX III

Reconstitution/Wash of Lyophilized Red Blood Cells at Study Site

Cryopharm personnel will be at the study site to supervise preparation of the samples prior to actual transfusion. All buffer solutions for reconstitution will be prepared in the class 100 clean room at Cryopharm. These buffer solutions will be tested for sterility and pyrogeniety by Cryopharm's Quality Control prior to release for clinical use. All buffer components are familiar reagents and most are commercially available as USP grade. The dried sample removed from the refrigerator will be allowed to warm up to room temperature before reconstitution. The reconstitution and cell washing will require a Cobe 2991 cell washer. The Cobe processing set will be installed according to the Operator's Handbook 2991. The dried cells will be reconstituted by adding sterile, reconstitution buffer (37°C). The reconstituted cells will then be washed several times to remove all cryoprotective reagents. It will be washed once with wash buffer for 15 minutes, followed by one wash with isolation buffer for two minutes, and two more washes with a Buffered Dextrose Saline-based solution for five minutes at each wash. The total processing time must be less than two hours. The unit will be accepted for transfusion if the hemoglobin is less than 200 mg/dl, total processing time less than three hours and unit meets specifications. The recovery, indices and osmotic stability assay will be performed on the lyophilized cells.

Labeling of Red Blood Cells
(Use Asceptic Technique at ALL Times)

1. 30 to 35 ml of washed, reconstituted lyophilized erythrocytes (RBC) are added to an empty sterile vial. Use a sterile air vent to vent the vial before injecting the red blood cells.

2. 5 ml of RBC are injected into a purple top tube to be used as the <u>background</u> control.

3. ^{51}Chromium (150 to 200 microcuries) are added to the vial which is gently swirled to maintain the RBC in suspension.

4. Incubate at 37°C for 15 minutes; swirl the vial gently on occasion.

5. Add ascorbic acid (100 mg) to the vial.

6. Draw 5 ml of labeled RBC into a hypodermic syringe, and inject into a purple top tube for <u>standard</u> control.

7. Draw 20 to 25 ml of labeled RBC into a fresh hypodermic syringe.

8. Weigh the filled syringe and empty 18-19 gauge butterfly set.

9. Attach the butterfly needle on the filled syringe and inject all of the labeled RBC into the patient within 30 minutes of labeling; RECORD TIME! The time of infusion should be less than one minute.

10. Weigh empty syringe and butterfly set after injection of blood.

11. Withdraw blood specimens into purple top tubes from opposite arm at timed intervals (refer to Section 5.4.5).

Red Cell Survival By Extrapolated Time Zero Method

1. Inject labeled RBCs into patient at Time Zero.

2. Obtain 5 ml blood samples from contralateral arm at timed intervals per protocol (Section 5.4.5).

3. Two aliquots of 1 ml from each sample are placed in 5 ml test tubes which are then capped, mixed and stored at 4°C until counting in a gamma counter. The samples collected during the first hour post injection will be counted on Day 1, then all samples will be counted after collection of the final sample on Day 5.

4. After collection of the 5, 7.5, 12 and 15 minute and one hour timed blood samples on Day 1, the duplicates of these time points will be counted for 10-30 minutes each in a gamma counter, along with the duplicate background and standard controls. The cpm in these samples will be used to calculate a preliminary extrapolated time zero and cell survival. This preliminary estimate is needed to determine whether the collection of blood samples should continue on the succeeding four days. At this point Dr. Weinstein and Cryopharm's clinical coordinator will determine whether the volunteer needs to return to the hospital for the scheduled blood sampling on days 2, 3, 4, and 5. If further blood collections are not warranted, the volunteer will only return for the scheduled follow-up examination after discharge from the hospital at the end of 24 hours post-injection.

5. Correct the CPM of each timed sample for ^{51}Cr elution (~1%/day) by multiplying the CPM for each time point by 0.01d where d = day post transfusion. Add these calculated elution values to the observed cpm for their corresponding samples. Correct the cpm of each sample for background by subtracting the mean cpm of the two background samples. Use the elution and background corrected cpm to calculate extrapolated time zero and cell survival.

6. To determine the extrapolated time zero value, the mean corrected cpm of the duplicate 5, 7.5, 12, and 15 minute samples are plotted versus time, and an estimated time zero is obtained from the y intercept of a best straight line through the points.

7. Calculate percent recovery of label for each sample as R = (T/S) x 100 where R = % recovery, S = extrapolated Time Zero CPM, T = corrected timed sample mean CPM.

8. Determine apparent $T_{1/2}$ of ^{51}Cr survival by plotting % recovery as a function of time on semi-log paper and drawing the best straight line through the points (normal = 25 to 30 days).

9. If the study is continued through Day 5, all collected samples will be counted for 30 minutes each, and the extrapolated t_0 and cell survival recalculated as above, using all data points.

Red Cell Survival by Theoretical Time Zero Method

1. Make 1:10 and 1:100 dilutions of patient STANDARD in 0.9% saline.

2. Pipette 1 ml of the diluted specimens and of the 5 minute and 15 minute samples into each of two 5 ml test tubes.

3. Cap and mix test tubes. Count for 30 minutes in a gamma counter.

4. Total Injected Dose (cpm) = (A X B X C)

   where: A = CPM of 1 ml of STANDARD dilutions
   B = dilution factor of STANDARD
   C = weight of blood injected into patient (in grams) / 1.090 gram per ml

5. Theoretical Time Zero = $\frac{\text{Total Injected Dose}}{\text{Whole Blood Volume}}$

   Where whole blood volume is estimated as shown below.

6. Calculate percent recovery of label for each sample as R = (T/S) x 100 as before, substituting the theoretical time zero value for S

7. Calculate apparent $T_{1/2}$ as before using the % Recovery derived from theoretical time zero.

8. The values for extrapolated t_0 and theoretical t_0 should agree within 10%; otherwise, the $t_{1/2}$ in the study is suspect because rapid sequestration of labeled red cells can produce a falsely low extrapolated t_0 value (y intercept).

Whole Blood Volume

1. Calculate the predicted whole blood volume (WBV) according to the formula:

(for men) $WBV = 0.3669H^3 + 0.03219W + 0.6041$

(for women) $WBV = 0.3561H^3 + 0.03308W + 0.1833$

where: H = height in meters
W = weight in kilograms
WBV = Whole Blood Volume in liters