(2)

SECOND ANNUAL REPORT

February 1, 1990 - January 31, 1991

"Evaluation of Dried Storage of Platelets and RBC for Transfusion: Lyophilization and other Dehydration Techniques"

Grant No. N00014-89-J-1712
from the Office of Naval Research:
Navy Medical Research and Development Command
Department of the Navy

Performance Sites and Investigators:

East Carolina University School of Medicine;
Arthur P. Bode, Principal Investigator

University of North Carolina at Chapel Hill;
Marjorie S. Read, Robert L. Reddick, Investigators

American Red Cross Blood Services - Tidewater Region;
Stein Holme, W. Andrew Heaton, Investigators

Respectfully submitted, March 25, 1991

INTRODUCTION

This project is based on the empirical development of processes whereby blood platelets and erythrocytes can be stabilized to withstand lyophilization for long-term dried storage as a transfusion resource for combat casualty care. The work is being carried out at three collaborating medical centers: in Greenville, NC, Chapel Hill, NC, and Norfolk, VA. The investigators bring together expertise in lyophilization techniques, blood banking practice, platelet physiology and liquid storage studies, and blood cell survival assessments.

The second year of this grant was focused on three major efforts: extensive evaluation of paraformaldehyde-stabilized platelets (para-plts), development of new stabilization protocols ($KMnO_4$, etc.), and application of new and existing stabilization protocols to RBC. The para-plts are apparently well-stabilized against membrane re-arrangements or lysis potentially induced by the stresses of dehydration and rehydration. The presumed draw back of this approach was thought to render the platelet inert or incapable of hemostatic function. Our data have proven otherwise: para-plts have been shown to adhere to subendothelium ex vivo, promote coagulation, change shape in a manner related to activation of fresh platelets, and circulate for several hours or more in vivo. In the development of a permanganate-based protocol, we have found that a short incubation of platelets with 0.1% (final conc.) $KMnO_4$ seems optimal, but a protective agent such as trehalose, sucrose, or albumin must be added before dehydration to prevent disruption. For either dehydration protocol, the optional rehydration conditions are still under investigation, and it appears that the Uni-sol formulation developed by Drs. Holme and Heaton for liquid storage of platelets and RBC provides certain benefits. Adaption of these protocols to RBC

preparations has produced variable results; membrane ghosts appear in most preparations. Separate approaches to RBC stabilization are needed. The attached individual Results sections provide more detail on these findings as gathered at each performance site.

PUBLICATIONS AND PATENTS

Several publications of this work have been made in abstracts and conference presentations:

(1) "Rehydrated Platelets Maintain Hemostatic Properties". M.S. Read, R.L. Reddick, A.P. Bode. Federation Proceedings 4:4436, 1990.

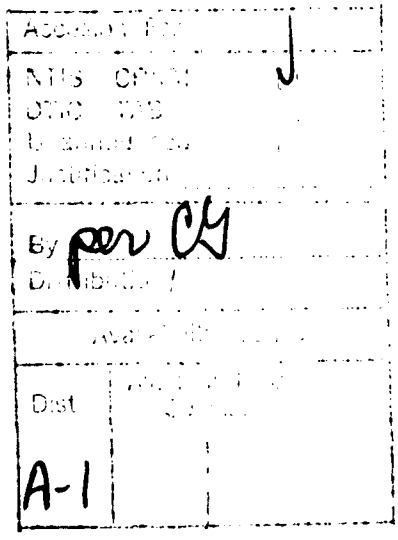
(2) "Stabilization of Platelet Membranes for Lyophilization and Dried Storage", A.P. Bode, M.S. Read, R.L. Reddick. AABB abstracts 1990: p. 138.

(3) "Exploratory Research in the Lyophilization of Blood Products", A.P. Bode and M.S. Read; presented at the US Naval MRDC Blood Research Program, March 14, 1990.

(4) "Studies with Dried and Rehydrated Platelets for Transfusion Products", M.S. Read, A.P. Bode, R.L. Reddick. Federation Proceedings 5:3093, 1991.

In addition, experimental data have been discussed at each investigator's institution in grand rounds or other formats. The feedback and critique by colleagues has been helpful. Manuscripts are now in preparation at UNC-Chapel Hill and at ECU for submission in the next few months to hematology journals. Also, submissions of abstracts of current data are planned for the 1991 meeting of the American Association of Blood Banks, and/or the American Society of Hematology.

The animal model trials and optimization of stabilization procedures currently underway will likely verify a patentable preparation protocol. As an interinstitutional agreement is drawn up, copies will be forwarded to the Naval MRDC for approval. At present, it appears that two or more protocols may be applied for coverage for platelet preparations. We are presently expanding efforts to develop a separate protocol for RBC. We have already observed the need for significant changes in adapting for RBC the procedures developed for platelets. Work is progressing in both areas.



RESULTS - Performance Site = East Carolina University

Paraformaldehyde Stabilization

In addition to the protocol developed by Dr. Read and coworkers in Chapel Hill, we have investigated the use of 0.2% - 0.4% paraformaldehyde under various conditions to minimize fixation effects. The lower paraformaldehyde concentration appeared to produce less clumping of platelets, but this difference was minimized by longer incubation times (up to 40 minutes). By immunofluorescence studies with flow cytometry, platelet surface glycoproteins Ib and IIbIIIa were well-preserved (92-96%). By Nomarski enhanced light microscopy, the morphology of para-plts appeared to be relatively intact except for a varying degree of degranulation.

In the context of para-plts, we found that the addition of platelet activation inhibitors (Prostagladin E-1 and theophylline) to the citrate anticoagulant used in blood collection improved the yield of platelets at the end of processing to 52% (versus 39% in controls) and reduced degranulation appreciably. This finding demonstrates the potential for platelet activation during blood processing, but incorporation of activation inhibitors into routine processing would not be readily practical. Final yields were improved also by inclusion of albumin during platelet washing and trehalose during the lyophilization step (74% recovery under best conditions).

These modified para-plts are being evaluated in the collaborating laboratories. Results are not yet consistent, but encouraging. Whether or not trehalose is advantageous in this protocol has yet to be decided. Vesiculation of platelet membranes is still evident by flow cytometry in the rehydrated preparations, but the effect of the resultant membranous microparticles on the functionality of these preparations is not known.

Rehydrated para-plts, from these modified processes or from the standard preparations produced in Chapel Hill, are currently under investigation by Dr. Read and colleagues for cell survival studies in circulation in experimental animals. A flow cytometry-based assessment is being developed with fluorescent membrane dyes to trace the infused cells in samples of peripheral blood taken at selected time points. Potentially, two-color analysis could be performed so that the cohort of platelets remaining after infusion can be examined for distinguishing features (such as lack of neo-antigens of platelet activation or appropriate expression of major surface glycoproteins like GPIb and GPIIbIIIa).

Permanganate Stabilization

Potassium or Sodium permanganate ($KMnO_4$ or $NaMnO_4$) was used in electron microscopy during its developmental period in 1950-1960 to prepare tissues for dehydration and infiltration with plastic for sectioning. Permanganate was found to be particularly well-suited to stabilization and highlighting of membrane features (Luft, J.H.; Biophys. Biochem. Cytol. 2:799-802, 1956), but was not described as a true fixative (Bradbury and Meek; Quart. J. Microscop. Sci. 101:241-250, 1960). We have employed weak solutions of permanganate (0.1% or 0.05% final conc.) with washed platelets and RBC to develop an alternative to

treatment with paraformaldehyde. For the past 6 months, our experimentation has focused on the varying effects of permanganate concentration, duration of incubation, pH of the buffer, presence or absence of albumin in the washes, and the need for other stabilizers (trehalose, sucrose, albumin) during lyophilization.

In the pilot experiments with 0.1% $KMnO_4$, we found that the washing buffer for platelets was optimal at near physiologic pH=7.3. The inclusion of theophylline in the first washing steps reduced subsequent clumping. A methodical approach could then be taken to other parameters in processing. An incubation time for platelets of 10 minutes in 0.1 or 0.05% $KMnO_4$ at R.T. proved sufficient to permit reaction of the permanganate with the cell suspension. However, platelet yields and integrity after lyophilization were greatly improved by the addition of 1M trehalose or sucrose or 0.1% human serum albumin to the resuspension buffer before dehydration. Typical experimental data included final yields of platelets (relative to the initial platelet-rich plasma before processing) of 97% for 0.05% $KMnO_4$ followed by 1M trehalose, 74% for 0.1% $KMnO_4$ followed by 1M trehalose, 40% for 0.1% $KMnO_4$ without further stabilization, and 23% for 0.05% $KMnO_4$ alone. Preservation of platelet membrane glycoprotiens was equivalent to that with paraformaldehyde (75-95% GPIb remaining); vesiculation of membranes during processing with $KMnO_4$ however, was only 1/10th-1/20th of that noted with para-plts. In similar experiments with 1M sucrose in the buffer during dehydration, GPIb preservation and platelet yields were good, and the percentage of discoid platelets rose to nearly 50%. Morphology was well-preserved also when 0.1% albumin was employed instead of trehalose or sucrose in the dehydration step.

Optimization of permanganate incubation conditions is still on-going. It appears that trehalose or sucrose or albumin provide essential protection during lyophilization after permanganate treatment. Optimal concentrations of these stabilization enhancers are still being worked out; with trehalose it appears that 1M > 0.5M > 0.25M > 0.1M in terms of platelet yield and integrity. Under the best empirical conditions to-date, rehydrated permanganate-treated platelets appear to produce micro-aggregates when stimulated with 10 μM ADP, or 1 mg/mL ristocetin, or 8 μg/mL collagen, and they have demonstrated the ability to adhere to glass slides and change shape under the light microscope. These preparations will be further evaluated by the collaborating investigators.

Other Progress Areas and Future Directions

Dried para-plts are being stored under various conditions (ex. -70°C freezer, ambient desiccator) to begin long-term evaluations of preparation stability. Similar conditions will also be applied to permanganate-treated platelets and to stabilized RBC as these protocols are worked out. Then, at selected monthly intervals, samples will be rehydrated and analyzed. Results will be compared to those preparations rehydrated within 72 hours of lyophilization.

Platelet activation inhibitors appear to improve platelet yields during washing and dehydration (see above). In a related project, we have made a study of cyclic AMP-active compounds in preserving liquid-stored platelet concentrates (manuscript in preparation). The conclusion reached so far is that a sustained elevation of cAMP above a threshold of approximately 50 pmoles/10^9 plts produced a long-lasting inhibition of platelet activation and preservation of platelet integrity/function for 15-20 days at room temperature in PL-732 storage containers. Information gathered in these studies will be applied as needed in stabilization protocols for dried platelet storage.

Stabilization of platelet and RBC membranes for frozen storage without drying has been assessed as an adjunct to protocol development for lyophilization. Instead of freeze-drying all the vials of a stabilized platelet or RBC preparation, we have frozen wet samples at -70°C and then thawed them out in the liquid state for analysis. In brief, we have found that with modified para-plts, thawed platelet yields were 30-60% when 1M trehalose or sucrose was present. Lower concentrations or absence of trehalose resulted in aberrant platelet shapes and poor recoveries in vitro. For RBC, concentrations of <400 mM trehalose were necessary after paraformaldehyde stabilization to prevent complete rupture following thawing. Typical RBC in vitro yields achieved were 10-50%. Further work in this area has been deferred because of the significant successes experienced with the dried and rehydrated preparations.

Vesiculation of blood platelets during in vitro liquid storage appears to be an irreversible manifestation of the platelet storage lesion (Bode et al., Blood 77:887-895, 1991). Membranous vesicles bearing platelet antigens have been observed by flow cytometry in most of the rehydrated platelet preparations tested to-date. We continue to refine the flow cytometry-based technique developed in this laboratory to enumerate and characterize these microparticles. Our findings could be very important in generating an understanding of how freeze-dried platelets are able to adhere to subendothelium or promote hemostatsis. We have related these microparticles to procoagulant and "pro-adherent" properties of liquid-stored platelets in related work. The potential capability of rehydrated platelets to vesiculate when appropriately stimulated will be studied, in addition to characterizing the function or effects or membranous microparticles present in the preparation at the time of rehydration. As these techniques become more refined, the surprising functionality of rehydrated platelets may be more easily explained.

SECOND ANNUAL REPORT
University of North Carolina at Chapel Hill

Feburary 1, 1990 - January 31,1991

Grant No. N00014-89-J-1712 from:
The Office of Naval Research
Department of the Navy

Evaluation of Dried Storage of Platelets and RBC for Transfusion: Lyophilization and Other Dehydration Techniques.

P.I. Arthur Bode, Ph.D. East Carolina University, Greenville North Carolina

Contract:
Dehydration of Platelets and RBC; Long-Term Storage of Transfusion Products.

P.I. Marjorie S. Read, Ph.D., University of North Carolina- Chapel Hill, North Carolina.

Co. P.I. Robert L. Reddick, M.D., UNC-CH

Submitted March 11, 1991

Platelet adhesion to the vascular wall is one of the first steps in primary hemostasis. Adhesion requires three conditions,1) normal vascular subendothelium, 2) von Willebrand factor (VWF), and 3) a normal platelet membrane.

Studies conducted during the past year.

Previous work showed that rehydrated platelets were capable of agglutinating, accelerating clotting of platelet poor plasma, adhering to subendothelium and binding fibrinogen.

During the past year we have continued to refine dehydration/rehydration protocols in order to produce a dried product that would adhere to subendothelium and exhibit characteristics of normal fresh platelets. We have continued to study rehydrated platelets for hemostatic properties as evidenced by morphologic change, following adhesion and the ability to form platelet-fibrin thrombi.

We have begun in vivo animal studies with rehydrated platelets to determine the half-life and in vivo characteristics of dried-rehydrated platelets.

A. The above investigators have developed dried and rehydrated platelets that will:

1. Adhere to vascular subendothelium.

In order to study the ability of rehydrated platelets to adhere to subendothelium, human and animal platelets were studied. The pig and dog were used since these are the animals we expect to use in animal studies prior to human trials. We have found species differences in platelet response in the annular perfusion chamber. These differences are of critical importance when choosing an animal model for pre-human trial testing. It is imperative that we determine the best animal model for in vivo testing.

Platelets from each species were isolated, washed and stabilized by treatment with aldehydes. Paraformaldehyde and glutaraldehyde were compared as stabilizing agents. A short fixation time of 60 minutes is best if paraformaldehyde is present and 20 min fixation with very low concentrations if glutaraldehyde is used. These conditions give the most active platelet thus far in our study. Higher concentrations of glutaraldehyde will lyse the cells and longer exposure times to either agent is unnecessary. However, treatment of cells with paraformaldehyde can be overnight at 4 degrees with good platelet response. The advantage of a longer and slower stabilization time with paraformaldehyde is convience and not one of platelet response.

Following fixation, the platelets were washed free of aldehyde with buffered saline. Test for presence of aldehydes following the washes have been negative. Albumin

was added to the stabilized platelets and the mixtures frozen and lyophilized to dryness.

Adhesion to subendothelium was measured by circulating whole blood across everted vessel segments and determining the number of platelets adhering to the deendothelized surface. The vessel segments are examined by electron microscopy and the number of platelets adhering and the morphology of the adherent cells is recorded. We are able to use this technique to evaluate the rehydrated platelets by removing the fresh platelets from whole blood and substituting an equilivant concentration of rehydrated platelets. Controls of whole blood depleted of platelets and without added rehydrated platelets as well as normal whole blood pumped across vessel segments are compared to the test vessel for numbers of adhering platelets. The presence of pseudopodia and degree of platelet spread are noted and recorded.

In the annular perfusion chamber, human and porcine rehydrated platelets adhered to the subendothelium of respective homologous vessels as well as fresh platelets. The numbers of platelet did not significantly differ for either species when rehydrated platelets replaced fresh platelets in the whole mixture. Interestingly, there was marked decrease of adhering canine platelets either fresh or rehydrated. The number of canine fresh platelets versus number of canine rehydrated platelets was not different, but the response between species was. These data indicates that the porcine may be the animal of choice for in vivo trials.

2. Form pseudopodia and undergo spread.

Platelet shape change is mediated by an energy-dependent proces. ADP, collagen, thrombin, and aracodonic acid all induce platelet shape change. Human, porcine and canine rehydrated platelets were studied by scanning electron microscopy (SEM) in order to investigate if stabilized platelets would respond to stimuli. Rehydrated platelets were layered on formvar-coated grids and examined by SEM for pseudopod production and spread. Rehydrated platelets were compared to fresh platelets as to the number of pseudopodia per platelet and the ability to form pancake-like arangements on the grids. All rehydrated platelets which had been stabilized with paraformaldehyde had multiple pseudopodia and many of the platelets were spread along the grid. To the contrary, platelets stabilized with glutaraldehyde often did not form pseudopodia and remained in the spherical form with few platelets spread. There was no marked differences between species of platelets as to shape change response.

These data indicate that paraformaldehyde may offer advantages over glutaraldehyde as a stabilizing agent. Glutaraldehyde may cause the membrane to "harden" to the extent that the cell is not able to respond to surface

activating stimuli. Futher work is needed to differentiate the advantages of these two stabilizing agents. The degree of "hardening" or cross-linking of membrane proteins may play a critical role in allowing the rehydrated platelet to mimic fresh platelets in shape change reactions.

C. Remain in circulation for at least two hours.

To determine if rehydrated platelets will circulate we chose the dog model for ease of use. Glutaraldehyde treated platelets display autofluorescence, making them identifiable in the presence of fresh platelets. Thus, glutaraldehyde becomes a marker for platelets stabilized by that agent. WE selected a dog, calculated the total platelet volume and concentration, and prepared enough glutaraldehyde treated platelets to equal 10 % of the total fresh platelets. The platelets were infused into the dog and blood samples collected at selected intervals. Samples were shipped to Dr. Bode for flow cytometric analysis and identification of circulating glutaraldehyde platelets. None were present in any of the samples.

These data suggest that glutaraldehyde stabilized platelets may not be suitable as a transfusion product.

Secondly we prepared paraformaldehyde treated platelets which were labeled with a fluorescence dye. This dye, developed by Cynaxis, Cell Science, Inc.is taken up by the platelet and is not released, nor does it leak from the platelet. Again, an animal was selected and treated with a vlume of rehydrated, labeled, platelets equal to 10% of the original volume of fresh platelets. Samples were collected at given periods and analyzed by fluorescent microscopy for labeled rehydrated platelets. Circulating rehydrated platelets were detected in blood smears, platelet rich plasma preparations and/or platelet pellets from blood samples collected up to two hours. Samples collected at six and eight hours showed no labeled, rehydrated platelets present.

These data suggest that rehydrated platelets, as presently prepared, will circulate for short periods of time and predict that preparations may be made that will circulate for longer times, perhaps to a near normal life span. These data again indicate that paraformaldehyde stabilized platelets are preferable to those stabilized with glutaraldehyde.

It is important that we continue in vivo experiments with different preparations of stabilized platelets in order to prepare a product with a normal circulating time. Platelets from future infusions will be analyzed by Dr. Bode by flow cytometry for relative concentrations and for functional activities. Dr. Bode's laboratory will also examine sample preparations for indication of metabolic functional activities as well as cofactoral activities. That is, to determine if rehydrated platelets will suplement fresh platelet in hemostatic functions. Will rehydrated

platelets act in conjunction with reduced numbers of fresh platlets to cause normal agglutination in response to activating agents ADP, collagen and thrombin?

B. We have successfully prepared red blood cells (RBC) which can be dried and rehydrated with greater than 80% recovery.

During the past year each protocol developed for drying and rehydrating platelets was also applied to RBC. Contrary to the findings with platelets, glutaraldehyde appears to be the fixative of choice for RBC. Stabilized RBC have been frozen, dried and rehydrated in the presence of purified protein or homologous plasma. The dried RBC have been stored at -20 degrees and -70 degrees before rehydration. Rehydration has been with buffered saline and the rehydrated cells examined by phase microscopy.

To date the best preparation was with RBC stabilized for 30 minutes with glutaraldehyde and snap frozen in the presence of albumin or plasma. The rehydrated RBC appeared normal under phase examination and the recovery of whole RBC as compared to the total number of frozen cells was greater than 80%. We have not tested these cells for deformability as yet. Samples are being readied to be sent to Drs. Bode and Holmes for fragility and deformability studies prior to in vivo trials in an animal model.

It is clear that dried RBC can be prepared that can be reconstituted with excellent recovery rates. In vivo tests to determine if these cells will remain in circulation will be started in year three.

C. We have continued to investigate the use of microwave irradiation as a means to stabilize platelet membranes prior to freezing and drying.

Microwave heat stabilization remains a possibility as a non-invasive means to "harden" or stabilize membranes prior to freezing. To date we have not had a preparation of microwave treated cells that have responded to rehydration and testing as well as cells that have been aldehyde preserved.

Significance of these findings:

It is clear that dried and rehydrated blood cells can be prepared that retain some hemostatic and metabolic functions. These protocols, when fully developed, give promise of a transfusion product of blood cells that can be stored in the dry state. Such a product will revolutionize blood storage and emergency blood transfusion treatments.

Brief summary of plans for the comming year.

A. We will continue studies of a dried/rehydrated platelet product and ways to extend the time of in vivo survival.

B. We will continue to develop dried/rehydrated RBC preparations and begin in vivo half-life studies.

C. We will perform in vivo thrombosis studies designed to determine the thrombogenic potential of transfused rehydrated platelets.

Publications:

Studies with Dried and Rehydrated platelets for Transfusion Products. M.S.Read, A.P.Bode and R.L.Reddick,

American Red Cross

Mid-Atlantic Regional Blood Services
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700 Olney Road
Norfolk, Virginia 23507
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March 19, 1991

Dr. Arthur P. Bode
East Carolina University
School of Medicine, Dept. of Clin. Pathology
and Diagnostic Medicine
Attn: Platelet Research - Brady IS-08
Greenville, NC 27858-4354

Re: USN Grant No. N00014-89-J-1712

Dear Art:

Enclosed is a progress report and data on our studies to date with respect to the above referenced project. Also enclosed is a proposal for work to be done by the Research Department here in Norfolk during the third year (1991-1992) of this grant.

If you need any further documentation or have any questions, please let me hear from you as soon as possible.

Yours sincerely,

Stein Holme, Ph.D.
Scientific Director

SH:adg

enclosures

PROGRESS REPORT ON SAMPLES RECEIVED MARCH 5, 1991

Summary

(see enclosure for detailed testing of the samples)

RBC

The red blood cell samples contain few, less than 5 %, nonhemolyzed cells. Samples fixed with $KMnO_4$ showed membrane staining of this compound.

PLATELETS

Samples fixed with $KMnO_4$ showed marked clumping with few, less than 10 %, discoid platelets (an exception was samples to which trehalose had been added). Since the platelet counts in these samples were less than $0.200x10^6$, these were not tested for hypotonic shock response.

Samples fixed with paraformaldehyde showed best preservation of morphology and had little clumping. Some samples showed response to shape change with ADP and to hypotonic shock after being washed and resuspended in citrated plasma. This suggests that the platelets are still viable with intact metabolism. Sample with best functional response was sample 910124, fixed with 2 % paraformaldehyde and with 1 M trehalose for 40 min.

IN VITRO TESTING OF LYOPHILIZED FROZEN RBC AND PLATELETS

Platelets

All liquid samples were thawed out. If lyophilized, 1 mL of the platelet storage medium Unisol was added for resuspension. Platelet count, size distribution, and morphology scoring were then performed. Hypotonic shock response and extent of shape change were performed if the platelet count was $.3 \times 10^6/\mu l$ or greater.

A. Samples where only microscopic examination, platelet counts, and platelet size distribution were performed:

1. Sample 910201. 0.5% $KMnO_4$ 1M Sucrose.
   Platelet count - $.2258 \times 10^6/\mu l$
   Mean platelet volume - 4.027147 μ^3
   Mean platelet diameter - 1.973935 μ

   Morphology scoring: 160
   Disc(4pts.)=10 Sphere(2pts.)=35
   Dendrite(1pt.)=55 Balloon(0pt.)=0

2. Sample 910129. 0.5% $KMnO_4$.
   Platelet count - $.0846 \times 10^6/\mu l$
   Mean platelet volume - 5.398091 μ^3
   Mean platelet diameter - 2.176442 μ

   Morphology scoring: 141
   Disc(4pts.)=3 Sphere(2pts.)=33
   Dendrite(1pt.)=63 Balloon(0pt.)=1

3. Sample 910129. 1.0% $KMnO_4$.
   Platelet count - $.0362 \times 10^6/\mu l$
   Mean platelet volume - 5.723727 μ^3
   Mean platelet diameter - 2.219355 μ

   Morphology scoring: 129
   Disc(4pts.)=3 Sphere(2pts.)=21
   Dendrite(1pt.)=75 Balloons(0pt.)=1

4. Sample 910129. 1.0% $KMnO_4$ 1M Trehalose.
   Platelet count - $.1456 \times 10^6/\mu l$
   Mean platelet volume - 4.178949 μ^3
   Mean platelet diameter - 1.998432 μ

   Morphology scoring: 187
   Disc(4pts.)=12 Sphere(2pts.)=51
   Dendrite(1pt.)=37 Balloon(0pt.)=0

5. Sample 910305. 0.5% $MnNaO_4$ HSA.
   Platelet count - $.1578x10^6/\mu l$
   Mean platelet volume - 5.276215 μ^3
   Mean platelet diameter - 2.15994 μ

   Morphology scoring: 156
   Disc(4pts.)=8 Sphere(2pts.)=35
   Dendrite(1pt.)=54 Balloon(0pt.)=3

6. Sample 910305 1.0% $MnNaO_4$ HSA.
   Platelet count - $.0606x10^6/\mu l$
   Mean platelet volume - 6.965248 μ^3
   Mean platelet diameter - 2.369441 μ

   Morphology scoring: 144
   Disc(4pts.)=4 Sphere(2pts.)=32
   Dendrite(1pt.)=64 Balloon(0pt.)=0

7. Sample 910305 1% $MnNaO_4$ 1M Trehalose.
   Platelet count - $.1948x10^6/\mu l$
   Mean platelet volume - 5.418053 μ^3
   Mean platelet diameter - 2.179122 μ

   Morphology scoring: 157
   Disc(4pts.)=9 Sphere(2pts.)=35
   Dendrite(1pt.)=51 Balloon(0pt.)=5

B. Samples where platelet count, size distribution, morphology scoring, hypotonic shock response, and extent of shape change were done. The samples were washed twice with Unisol and Anticoagulant Citrate Dextrose solution (ACD) and resuspended with platelet-poor plasma for hypotonic shock response and extent of shape change testing:

8. Sample 910305 0.5% $MnNaO_4$ 1M Trehalose.
   Platelet count - $.5172x10^6/\mu l$
   Mean platelet volume - 4.5367 μ^3
   Mean platelet diameter - 2.0539 μ

   Morphology scoring: 165
   Disc(4pts.)=12 Sphere(2pts.)=31
   Dendrite(1pt.)=55 Balloon(0pt.)=2

   Hypotonic shock response=40% recovery
   Extent of shape change=3.94% increase O.D.

9. Sample 910124 2.0% Paraformaldehyde 40 minutes.
   Platelet count - $2.2004 \times 10^6/\mu l$
   Mean platelet volume - 5.8466 μ^3

   Mean platelet diameter - 2.2351 μ

   Morphology scoring: 314
   Disc(4pts.)=62 Sphere(2pts.)=28
   Dendrite(1pt.)=10 Balloon(0pt.)=0

   Hypotonic shock response=100% recovery
   Extent of shape change=0% increase O.D.

10. Sample 910124 2.0% Paraformaldehyde 1M Trehalose 30 minutes.
   Platelet count - $.6082 \times 10^6/\mu l$
   Mean platelet volume - 11.99007 μ^3
   Mean platelet diameter - 2.839712 μ

   Morphology scoring: 270
   Disc(4pts.)=40 Sphere(2pts.)=50
   Dendrite(1pt.)=10 Balloon(0pt.)=0

   Hypotonic shock response=0% recovery
   Extent of shape change=2.73% increase O.D.

11. Sample 910124 2.0% Paraformaldehyde 1M Trehalose 40 minutes.
   Platelet count - $.569 \times 10^6/\mu l$
   Mean platelet volume - 11.16966 μ^3
   Mean platelet diameter - 2.773408 μ

   Morphology scoring: 200
   Disc(4pts.)=10 Sphere(2pts.)=69
   Dendrite(1pt.)=22 Balloon(0pt.)=0

   Hypotonic shock response=22.2% recovery
   Extent of shape change=7.19% increase O.D.

12. Sample 910124 2.0% Paraformaldehyde 30 minutes.
   Platelet count - $.6344 \times 10^6/\mu l$
   Mean platelet volume - 6.275055 μ^3
   Mean platelet diameter - 2.288440 μ

   Morphology scoring: 272
   Disc(4pts.)=38 Sphere(2pts.)=58
   Dendrite(1pt.) Balloon(0pt.)=0

   Hypotonic shock response=100% recovery
   Extent of shape change=1.57% increase O.D.

Red blood cells

All of the samples were thawed out or 1 ml. of the storage medium Unisol was added to the lyophilized samples for resuspension. The samples were examined under the microscope. The majority of the cells in all the samples were ghost cells with few remaining intact red blood cells.

1. Sample 901022 1M Trehalose 25mM KCl ETOH
   Less than 1% intact red blood cells (rbcs).

2. Sample 901022 800mM Trehalose 25mM KCl ETOH
   5% intact rbcs.

3. Sample 901016 250mM Sucrose 25mM KCl ETOH
   Less than 1% intact rbcs.

4. Sample 901016 250mM Trehalose 25mM KCl ETOH
   Less than 1% intact rbcs.

5. Sample 901022 800mM Sucrose 25mM KCl ETOH
   Less than 1% intact rbcs.

6. Sample 901022 1M Sucrose 25mM KCl ETOH
   1 to 2% intact rbcs.

7. Sample 910201 0.5% KMnO4 1M Sucrose
   5% intact rbcs.

8. Sample 910201 1.0% KMnO4 1M Sucrose
   Less than 1% intact rbcs.

PROPOSAL FROM THE ARC LABORATORY FOR THE THIRD YEAR

A. INVESTIGATION OF PHYSIOLOGIC, METABOLIC, AND FUNCTIONAL PROPERTIES OF LYOPHILIZED PARAFORMALDEHYDE-TREATED PLATELETS.

Background and objective.

A very promising finding last year was that lightly paraformaldehyde-fixed platelets tolerated lyophilization, and, after resuspension in citrated plasma, demonstrated well preserved morphology and maintenance of functional properties such as adhesion and a shape change response to the agonist ADP. This suggested that these platelets were still viable with intact metabolic and functional properties.

The main goal in the forthcoming year will be to establish the maximal exposure in time and concentration to paraformaldehyde of the platelets that is needed for lyophilzation, while still maintaining normal platelet physiologic, metabolic, and functional properties.

Protocol.

1 day old standard CPD-anticoagulated platelet concentrates (PC) will be obtained from ARC, Mid-Atlantic Region.

Samples of 5 mL PC will be exposed to different concentrations of paraformaldehyde (0.25 % - 2 %) in a phosphate/acetate-containing platelet storage medium (Setosol) for various periods of time (10-60 min) at 20-24 C°. A volume ratio of 1 part PC to 3 parts paraformaldehyde-Setosol will be used.

After the fixation, samples will be counted and sent to Dr. M. Read, Chapel Hill, for lyophilization. The lyophilized samples will be returned to the ARC laboratory in Norfolk for evaluation of platelet yield and metabolic and functional properties.

5 mL of Setosol will be added to the sample with resuspension of the lyophilized platelets. Platelet count and size distributio. will be performed using a Coulter counter to measure platelet yield.

In vitro assays.

The Setosol-suspended platelets will be washed once and resuspended in autologous CPD-plasma for in vitro testing. Comparison will be performed with stored standard CPD-anticoagulated platelet concentrates.

Platelet spreading on glass.

A small sample will be added to a microscopic slide to examine the ability of the platelets to spread on glass. This property involves the change of platelet morphology from smooth discs to a pancake appearance with pseudopods and requires the intactness of the platelet cytoskeleton and normal energy metabolism.

Platelet shape change, aggregation and secretion with collagen, ADP and thrombin.

These functional responses will be tested in a Chrono-log Aggregometer as described previously. Platelet shape change and aggregation will be determined by changes in light transmission. The release of ATP as measured by bioluminescence will reflect the ability of the platelets for secretion.

Platelet respiratory rate, glycolytic rate and levels of adenine nucleotides.

These measurements of platelet energy metabolism will be performed as described previously. Briefly, CPD-plasma-diluted PC (count: 300,000 plts/μL) will be transferred to an airtight syringe and incubated for 2 hours at 37°C in a water bath. Before and after incubation samples will be taken for measurement of oxygen uptake, glucose consumption, lactate production, and levels of ATP, ADP, AMP and hypoxanthine. pH, pO_2, pCO_2 levels will be determined with a gas analyzer, levels of lactate and glucose by standard enzymatic assays, adenine nuleotides and their breakdown product, hypoxanthine, by HPLC.

Platelet adherence to everted rabbit aorta.

As a measure of hemostatic effectivness, the lyophilized platelets' ability to adhere to everted rabbit aorta will be determined. Briefly, the platelets will be labeled with 111-In-oxine and resuspended in CPD-plasma using standard methods.

B. IN VITRO TESTING OF LYOPHILIZED RBC AND PLATELET SAMPLES SENT TO THE ARC LABORATORY FROM THE PRINCIPAL INVESTIGATORS.

RBC samples.

Rehydration of lyophilized red cell samples will be performed by addition of a red-cell suspending medium. Up to now we have not been sucessful in obtaining non-hemolyzed lyophilized RBC's. Part of the problem could be the resuspension medium. We, therefore, plan to investigate the suitability of various commercially available RBC additive solutions used in storage as resuspension medium. Once we have been able to obtain non-hemolyzed lyophilized RBC, testing will be performed to measure deformability by filtration and viability by levels of ATP.

Platelet samples.

In vitro testing of platelet samples will be performed as described above under section A.

C. IN VIVO PLATELET AND RBC SURVIVAL STUDIES

In the original proposal, it was planned to performed in vivo survival studies using human volunteers in the third year. As described in the original proposal, these will be conducted only after animal studies demonstrate that the lyophilized cells do not represent any danger to the animals and that the cells survive after the infusion.