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13. ABSTRACT ***(Maximum 200 Words)***

The Bionetics Corporation staffed and maintained facilities to manufacture a hemoglobin based blood substitute and to support red blood cell preservation and pathogen inactivation research for the Blood Research Detachment and Department, Walter Reed Army Institute of Research, at 1413 Research Boulevard, Rockville MD 20850 and later at Building 503, Walter Reed Forest Glen Annex, Silver Spring, MD 20910. During the operation of the hemoglobin production facility, 106.04 L of hemoglobin solution containing 11.32 kg of hemoglobin was manufactured, which met or exceeded contract specifications. Three specialty products were produced and the facility was prepared for indefinite shut down. Contract staff completed a series of *in vitro* experiments and clinical trials, which led to the development of red cell preservation systems extending the shelf life of refrigerated blood from 6 to 12 weeks and a US patent. Data from the laboratory resulted in the publication of 12 manuscripts and six abstracts in the peer-reviewed literature. Evaluation of two pathogen-inactivation technologies for eradication of infectious agents in blood products are in progress. The work done under this contract by the Bionetics Corporation helped advanced the WRAIR Blood Research mission.

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Table of Contents

E. "Eleven week blood storage," J.R. Hess, N. Rugg, J.F. Gormas, A.D. Knapp, H.R. Hill, C.K. Oliver, L.E. Lippert, E.B. Silberstein, and T.J. Greenwalt, Transfusion, 2001; 41:1586-1590.

F. "Maintaining RBC ATP for 12 weeks," J.R. Hess, H.R. Hill, C. Oliver, L.E. Lippert and T.J. Greenwalt, Vox Sanguinis, 2002; 83, S2: 109.

G. "Report on 12 week storage of red cells in a low chloride, alkaline, hypotonic additive solution," T.J Greenwalt, A. Knapp-Jones, N. Rugg, and J.R. Hess, Vox Sanguinis, 2002; 83, S2: 116.

H. "The effects of phosphate, pH, and additive solution volume on RBCs stored in saline-adenine-phosphate-glucose-mannitol additive solutions," J.R. Hess, L.E. Lippert, C.P. Derse-Anthony, H.R. Hill, C.K. Oliver, N. Rugg, J.F. Gormas and T.J. Greenwalt. Transfusion 2000; 40:1000-1006.

I. "The role of electrolytes and pH in RBC additive solutions," J.R. Hess, N. Rugg, A.D. Knapp, J.F. Gormas, H.R. Hill, C.K. Oliver, L.E. Lippert and T.J. Greenwalt, Transfusion 2001; 41:1045-1051

J. "Alkaline CPD and the preservation of red blood cell 2,3-DPG," J.R. Hess, H.R. Hill, C.K. Oliver, L.E. Lippert, T.J. Greenwalt, Transfusion 2002; 42:747-752.

K. "Effect of 24 hour storage at 25°C on the *in vitro* storage characteristics of CPDA-1 packed red blood cells," J.P. Ruddell, L.E. Lippert, J.G. Babcock, and J.R. Hess, Transfusion 1998; 38:424-428.

L. "The viability of autologous human red cells stored in additive solution-5 and exposed to 25^{0}C for 24 hours," T.J. Reid, J.G. Babcock, C.P. Derse-Anthony, H.R. Hill, L.E. Lippert and J.R. Hess, Transfusion 1999; 39:991-997.

M. "The effects of polyvinyl chloride and polyolefin blood bags on red blood cells stored in a new additive solution," H.R. Hill, C.K. Oliver, L.E. Lippert, T.J. Greenwalt and J.R. Hess, Vox Sanguinis, 2001; 81:161-166.

N. "A multicenter study of in vitro and in vivo values in human RBC's frozen with 40-percent (wt/vol) glycerol and stored after glycerolization for 15 days at 4°C in AS-3: assessment of RBC processing in the ACP 215," C.R. Valeri, G. Ragno, L.E. Pivacek, R. Srey, J.R. Hess, L.E. Lippert, F. Mettille, R. Fahie, E.M. O'Neill and I.O. Szymanski, Transfusion 2001; 41:933-939.

O. "The effects of two additive solutions on the postthaw storage of RBCs," J.R. Hess, H.R. Hill, C.K. Oliver, L.E. Lippert, and T.J. Greenwalt, Transfusion 2001; 41; 923-927.

P. "Buffered washing improves red cell post thaw storage," L.E. Lippert, M.J. Lopatka, H.R. Hill, J.M. Salata and J. Holmberg, Vox Sanguinis 2002; 83, S2:116-117.

Q. "Prestorage leukoreduction may reduce hemolysis of frozen/thawed red cells post deglycerolization," L.E. Lippert, M.J. Lopatka, H.R. Hill, J.M. Salata, J.A. Holmberg, Unpublished.

R. "Psoralen photochemical inactivation of *orientia tsutsugamushi* in platelet concentrates," K.J. Belanger, D.J. Kelly, F.C. Mettille, C.V. Hanson and L. E. Lippert, Transfusion 2000; 40:1503-1507.

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Introduction

Because combat is synonymous with bloodshed and blood replacement saves lives, the U.S. Army Medical Research and Materiel Command maintains facilities and programs to develop improved blood products. The purpose of this contract has been to operate and maintain research laboratory facilities and a hemoglobin production facility for the development of improved blood products for use by the military blood program. For the first two years of the contract and until the government discontinued the effort, contract staff operated and maintained a pilot plant scale production facility for the manufacture of a precision hemoglobin-based blood substitute or hemoglobin-based oxygen carrier (HBOC). Contract staff also operated and maintained research laboratories for development of red blood cell preservation systems, improved processes for glycerolization, deglycerolization and post-deglycerolization liquid storage of frozen blood and the evaluation of blood pathogen inactivation/reduction processes.

Narrative Description of Contract Activities and Results

Introduction

The final report for contract DAMD-17-C-94-4154, a services contract between the U.S. Army Medical Research Acquisition Agency and The Bionetics Corporation, will be organized around three major topics. Those topics are hemoglobin-based blood substitutes, red cell preservation, and pathogen inactivation. A brief narrative for each of the topics will be developed with published journal articles, abstracts and other written materials, which are appended to the report. The narrative is intended to be a summary, which leads the interested reader to the appendices for details of methods, research data, a thorough discussion of the results and conclusions with appropriate literature citations.

Hemoglobin-Based Blood Substitutes

The major contract activity during the first two years of contract operation was the operation and maintenance of the Hemoglobin Production Facility (HPF). During those two years, 106.04 liters hemoglobin solution containing 11.32 kilograms of hemoglobin and three specialty hemoglobin products were produced for use and distribution by the government. Concurrent with the HPF operation, the manufacturing process was systematically examined and a series of process improvements were instituted. The process improvements focused on incorporating and substituting US Pharmacopoeia (USP) materials for non-USP reagents, and streamlining the manufacturing steps to reduce process duration and maximize efficiency. The result of those process improvements increased purity by 30% and yield by 25%. The manufacturing process and process improvements instituted are described in a peer-reviewed publication, "An improved process for the production of sterile modified hemoglobin solutions," F.A. Highsmith, C.M. Driscoll, B.C. Chung, M.D. Chavez, V.M. Macdonald, J.M. Manning, L.E. Lippert, R.L. Berger and J.R. Hess, Biologicals 1997; 25:257-268. A copy of this article is attached as Appendix A.

Red Blood Cell Preservation - Introduction

When the contract began, the available Food and Drug Administration (FDA) licensed blood collection and storage systems allowed a maximum of six weeks storage at refrigerator (2 – 6 ^{0}C) temperature when stored continuously in the liquid state and only 24 hours after thawing and deglycerolization following frozen storage (-65^{0}C or colder). The initial goal was to develop a storage solution for liquid stored cells, which would allow a minimum of eight weeks of liquid storage and several days of storage of previously frozen red cells. The next two sub-sections of this report describe the results of research in developing extended storage systems, beginning with liquid stored cells. The military utility of these improvements in red cell storage would be to allow greater flexibility in the utilization of this scarce resource and potentially reduce resupply requirements by one-third.

Red Blood Cell Preservation – Liquid Stored Red Cells

The initial attempts at developing an extended storage solution were in collaboration with the MedSep Corporation, which had purchased rights to a hypotonic and low chloride storage solution, designated AS-24. This hypotonic storage solution had proven to reduce red cell morphological changes as refrigerated storage progressed. A morphology score, derived from microscopic examination of hundreds of red cells, which were in turn categorized into one of six morphological stages, was thought to be an *in vitro* surrogate for *in vivo* recovery and survival. The data from a series of *in vitro* tests and *in vivo* red cell survival and recovery studies with the hypotonic storage solution comparison to licensed systems was published in the journal Transfusion. A copy of that journal article, "A hypotonic storage solution did not prolong the viability of red blood cells," J.G. Babcock, L.E. Lippert, C.P. Derse-Anthony, M. Mechling and J.R. Hess, Transfusion 2000; 40:994-999 is appended to this report as Appendix B. Though it was not possible to demonstrate satisfactory *in vivo* red cell recovery at eight weeks, some insights into red cell preservation and metabolism were developed, which set the stage for later work. Furthermore, our laboratory developed the capability to perform autologous *in vivo* red cell survival and recovery measurements, a capability shared by less than ten laboratories nation wide. Before the contract concluded, our laboratory performed in excess of 100 autologous red cell recovery and survival studies, which provided data for six research protocols.

In collaboration with Tibor J. Greenwalt of the University of Cincinnati Hoxworth Blood Center, the preceding research was followed by the formulation and *in vitro* and *in vivo* testing of a series of experimental additive solutions (EAS). The early EAS formulations were built around shifting to an alkaline pH by substituting some or all of the monosodium phosphate (pKa 5.5) with disodium phosphate (pKa 8.5). In addition, the concentration of each of the other components of the FDA licensed additive was incrementally adjusted in a structured progression of experiments. The volume of additive solution to be added to a unit of blood was also incrementally increased for 100 mL to 200 mL and in some experiments, to 300 mL. The only ingredient not adjusted was the adenine concentration. Only after years of intense effort did the FDA allow addition of 2 mM adenine to red cell storage solutions; deviation from that concentration would only serve to greatly prolong regulatory approval.

The first result of that structured and disciplined approach was the development of EAS capable of storing red cells for nine weeks, ten weeks, eleven weeks and eventually twelve weeks at refrigerator temperatures with greater than 75% recovery at 24 hours post transfusion and less than 1% hemolysis. This is the unwritten standard established by the FDA for approval in the United States. The details of the nine, ten and eleven week storage solutions, the rationale for the EAS formulation, and results of *in vivo* red cell survival studies and preceding *in vitro* studies are provided in Appendices C, D, and E respectively; "Successful storage of RBCs for nine weeks in a new additive solution," J.R. Hess, N. Rugg, A.D. Knapp, J.F Gormas, E.B Silberstein, and T.J. Greenwalt, Transfusion 2000; 40:1007-1011; "Successful storage of RBCs for ten weeks in a new additive solution," J.R. Hess, N. Rugg, A.D. Knapp, J.F Gormas, E.B Silberstein, and T.J. Greenwalt, Transfusion 2000; 40:1012-1016; and "Eleven week blood storage," J.R. Hess, N. Rugg, J.F. Gormas, A.D. Knapp, H.R. Hill, C.K. Oliver, L.E. Lippert, E.B. Silberstein, and T.J. Greenwalt, Transfusion, 2001; 41:1586-1590. The data from the twelve-week storage solutions was only recently presented in abstract form at the September 2002 meeting of the International Society of Blood Transfusion. Copies of the published abstracts, "Maintaining RBC ATP for 12 weeks," J.R. Hess, H.R. Hill, C. Oliver, L.E. Lippert and T.J. Greenwalt, Vox Sanguinis, 2002; 83, S2: 109, and "Report on 12 week storage of red cells in a low chloride, alkaline, hypotonic additive solution," T.J Greenwalt, A. Knapp-Jones, N. Rugg, and J.R. Hess, Vox Sanguinis, 2002; 83, S2: 116, are included in this report at Appendices F and G respectively. Furthermore, the EAS formulation strategy was awarded a U.S. patent held jointly by the Walter Reed Army Institute of Research and the University of Cincinnati, with COL John R. Hess, the COR during the first six and one half years of the contract and Dr. Tibor J. Greenwalt as coinventors.

The data from the five years of research also led to several insights into red cell preservation and physiology. The roles of pH, salt concentrations, volume and phosphate were better understood from the data generated in a set of *in vitro* experiments. The results of those experiments and the insights in to red cell preservation are described in detail in a set of peer-reviewed publications at Appendix H, I, and J. Those publications are "The effects of phosphate, pH, and additive solution volume on RBCs stored in saline-adenine-phosphate-glucose-mannitol additive solutions," J.R. Hess, L.E. Lippert, C.P. Derse-Anthony, H.R. Hill, C.K. Oliver, N. Rugg, J.F. Gormas and T.J. Greenwalt, Transfusion 2000; 40:1000-1006; "The role of electrolytes and pH in RBC additive solutions," J.R. Hess, N. Rugg, A.D. Knapp, J.F. Gormas, H.R. Hill, C.K. Oliver, L.E. Lippert and T.J. Greenwalt, Transfusion 2001; 41:1045-1051; and "Alkaline CPD and the preservation of red blood cell 2,3-DPG," J.R. Hess, H.R. Hill, C.K. Oliver, L.E. Lippert, T.J. Greenwalt, Transfusion 2002; 42:747-752, respectively. The results in the last of these publications led to a crucial understanding of the delicate balance of pH required to maintain both ATP generation and 2,3-DPG levels. The levels of 2,3-DPG, and consequently the red cells' ability to unload oxygen at the tissues, ordinarily fall to very low levels after 5 – 7 days of refrigerated storage in nearly all storage systems tested because pH falls. Conversely, if pH is held at levels above 7.6, DPG is maintained but ATP is sacrificed. However, when pH is carefully maintained ATP generation can be maintained without greatly sacrificing DPG.

In addition to the biochemical aspects of red cell preservation, key biophysical aspects were investigated as well. The first was the effect of temperature on the red cell storage lesion. The issue of storage at ambient rather than controlled refrigeration temperatures comes from a very practical circumstance, refrigeration failure. The FDA mandated storage temperature for red cells is 2 – 6^0C; and, during transport the temperature is not to exceed 10^0C. Given the austere conditions under which the military medicine is often required to operate, it is not always possible to maintain those narrowly controlled conditions. Conversely, even under the best of conditions, refrigerators fail or refrigerator doors are left ajar for extended periods of time exposing the blood to temperatures in excess of the maximum allowed. Should that occur, questions regarding the safety and usability of the blood arise. "Is the blood safe to use?" "Should the blood be destroyed?" Little data existed describing the degree to which red cells are compromised when red cells are exposed to warmer temperatures. The degree to which the red cell storage lesion is accelerated was first examined in a set of *in vitro* experiments and the results published. A copy of that publication, "Effect of 24 hour storage at 25^0C on the *in vitro* storage characteristics of CPDA-1 packed red blood cells," J.P. Ruddell, L.E. Lippert, J.G. Babcock, and J.R. Hess, Transfusion 1998; 38:424-428, is included to this report as Appendix K.

The research described in the publication cited above was the first in which our laboratory employed a red cell pooling and splitting experimental design, a technique used extensively in subsequent *in vitro* experiments. It is also the first time, to our knowledge, that this technique was described in the published literature as being used for the study of red cell preservation. The process begins with collecting a set of whole blood units of identical ABO; the size of the set, ordinarily three or four, is determined by the number of experimental conditions being studied plus one for a control. The units in the set are pooled and then divided into the number of aliquots in the original pool. The individual aliquots are then assigned to the different treatment groups of the control. Additional pools are created to produce the replicates. The greatest advantage of this technique is to average the ordinarily encountered very significant differences among individual blood donor's response to storage and experimental conditions into each of the aliquots in the pool. Because the measured variance among samples in a pool are far less than among individual donors, far smaller sample sizes, and consequently fewer blood donors, are need to measure statistically significant differences between treatment groups. At the same time, the geometry and size of the single unit blood collection, a significant determinant of red cell preservation, can be maintained.

Based on the results of the *in vitro* experiments of storing blood at ambient temperatures, a clinical trial was undertaken. The *in vivo* autologous red cell 24-hour post transfusion recovery and survival was measured when the blood was exposed to 25^0C for twenty-four hours. The results were compared to blood continuously stored at 2 – 6^0C. The results of that clinical trial were published in the journal Transfusion. A copy of that manuscript, "The viability of autologous human red cells stored in additive solution-5 and exposed to 25^0C for 24 hours," T.J. Reid, J.G. Babcock, C.P. Derse-Anthony, H.R. Hill, L.E. Lippert, and J.R. Hess, Transfusion 1999; 39:991-997, is included in this report as Appendix L.

The second biophysical aspect of red cell storage studied was the effect of storage in an alternate plastic. The industry standard for red cell storage is a polyvinyl chloride (PVC) with a diethylhexyl phthalate (DEHP) plasticizer. The manufacturing of PVC generates several environmental hazards and the leaching of the DEHP into biological membranes has generated some health concerns. Paradoxically, though the DEHP is a health concern, the compound confers a degree of deformability and flexibility to the red cell membrane, which enhances their preservation in refrigerated storage. This research was initiated to determine if blood stored in an alternate plastic, polyolefin (PO), with the storage solution EAS-61, would allow storage for six weeks. The results of that research was published in the journal Vox Sanguinis, "The effects of polyvinyl chloride and polyolefin blood bags on red blood cells stored in a new additive solution," H.R. Hill, C.K. Oliver, L.E. Lippert, T.J. Greenwalt and J.R. Hess, Vox Sanguinis, 2001; 81:161-166, and is included in this report as Appendix M.

Red Blood Cell Preservation – Following Thawing and Deglycerolization of Previously Frozen Stored Red Cells

Until very recently, the maximum allowable storage of thawed and deglycerolized red cells following freezing was 24 hours. This limitation is imposed because the processing of the red cells during the glycerolization and deglycerolization stages occurs in an open environment. Because the connections to various solutions used during these processes are open to the ambient air, the possibility of airborne bacterial contamination exists. To minimize the potential of bacterial growth and subsequent harm to the transfusion recipient, the storage time is limited. A closed process system was recently developed, which would allow storage of the thawed, deglycerolized blood beyond 24 hours. Our laboratory was part of a multicenter evaluation of that system and contributed both to the *in vitro* evaluation and was one of two laboratories to perform *in vivo* autologous red cell survival and 24-hour post transfusion recovery of red cells processed in this system. The results of those evaluations were used to obtain FDA clearance for use in the United States and were also published. That publication, "A multicenter study of in vitro and in vivo values in human RBC's frozen with 40-percent (wt/vol) glycerol and stored after glycerolization for 15 days at 4^0C in AS-3: assessment of RBC processing in the ACP 215," C.R. Valeri, G. Ragno, L.E. Pivacek, R. Srey, J.R. Hess, L.E. Lippert, F. Mettille, R. Fahie, E.M. O'Neill and I.O. Szymanski, Transfusion 2001; 41:933-939, is included in this report as Appendix N.

Though the closed system processing of frozen blood now allows 15 day versus 24-hour post thaw storage, potential existed for even longer storage. The first set of those experiments to extend the post thaw storage limit employed the EAS-61 and the standard AS-3 in both 100 and 200 mL volumes. The results of that *in vitro* experiment were published, "The effects of two additive solutions on the postthaw storage of RBCs," J.R. Hess, H.R. Hill, C.K. Oliver, L.E. Lippert, and T.J. Greenwalt, Transfusion 2001; 41;923-927; and are included in the report as Appendix O.

One of the conclusions of the preceding set of experiments was that extended post thaw storage might be extended by reformulating the wash solution. To test that hypothesis, a similar experiment was performed in which the standard unbuffered wash solution, pH5.5, was compared to deglycerolizing the cells with a wash solution supplemented with 20 mM disodium phosphate. Two significant conclusions were obtained for those experiments. The first observation was that 1) the pH was maintained at higher levels for longer periods of time when processed with the buffered wash, 2) ATP production was enhanced when processed in the buffered wash, and 3) hemolysis remained below the 1% maximum at 42 days of storage. Taken together, those results indicate potential for storage beyond 15 days. Those results were reported at the 2002 meetings of the International Society of Blood Transfusion and were published in abstract form, "Buffered washing improves red cell post thaw storage," L.E. Lippert, M.J.

Lopatka, H.R. Hill, J.M. Salata and J. Holmberg, Vox Sanguinis 2002; 83, S2:116-117; a copy is attached to this report as Appendix P.

The second significant observation for those experiments was that prestorage leukoreduction filtration reduced post thaw hemolysis. Hemolysis was reduced by approximately 50% at 15 days post thaw storage when compared to the data from the initial evaluation of system, Appendix N, in which blood was not leukoreduced. This finding adds another dimension to the conventional understanding of the role of leukoreduction and the glycerolization, freezing and deglycerolization process. That process removes significant numbers of leukocytes and would thereby be expected to confer any beneficial effect without regard to the use of leukoreduction prior to freezing. These results would indicate a different conclusion. One interpretation of the data is that the leukoreduction must occur prior to freezing in order to confer any effect on post thaw storage. These finding will be presented in abstract form at the 2002 meetings of the American Association of Blood Banks. A copy of the submitted and accepted abstract, "Prestorage leukoreduction may reduce hemolysis of frozen/thawed red cells post deglycerolization," L.E. Lippert, M.J. Lopatka, H.R. Hill, J.M. Salata, J.A. Holmberg, is attached as Appendix Q.

Pathogen Inactivation

A significant research activity has been pathogen inactivation. Pathogen inactivation research has become a core funded research program for the Blood Research Department and has become the primary research focus during the last year of the contract. This work has been pursued primarily in collaboration with two commercial partners, Cerus Corporation and Gambro BCT within the context of Cooperative Research and Development Agreements (CRADA) between the commercial partner and the Institute. This research activity capitalizes on the greatest strength of the Institute, that being infectious diseases and our ability to form collaborations with infectious disease investigators. The first of those pathogen inactivation experiments was to determine the effectiveness of a photoactive chemical, a psoralen designated AMT, to inactivate the ricketssial organism responsible for scrub typhus. The result of that research was published and a copy of the publication, "Psoralen photochemical inactivation of *Orientia tsutsugamushi* in platelet concentrates," K.J. Belanger, D.J. Kelly, F.C. Mettille, C.V. Hanson and L. E. Lippert, Transfusion 2000; 40:1503-1507, is included in this report as Appendix R. Additional work with the psoralen technology will be continued after the contract ends. A set of validation experiments in which the effectiveness of a proprietary psoralen, S-59, to inactivate *Orientia tsutsugamushi* will be tested. The animal use protocol has been approved; the protocol will be initiated after the current staff receives training in the procedures and processes for handling a biosafety level 3 (BSL-3) organism and working in a BSL-3 laboratory.

The second pathogen inactivation technology evaluated utilizes the B vitamin, riboflavin (RF). The RF process is being developed by Gambro BCT. The RF technology has two appealing features. First, the material used to inactivate the organism is a natural product, a required constituent of human diets and its metabolites that are very well characterized. Second, the process can be used for both plasma and cellular blood products; the psoralen technology cannot be used with any red cell containing product because the UVA light needed to activate the psoralen is absorbed by hemoglobin. The initial evaluation of the RF technology was a test of its effectiveness in inactivating the malarial parasite, *Plasmodium falciparum*. Preliminary results indicate the process is effective against the parasite. Those results were reported at the 2002 Conference of the International Society of Blood Transfusion. An abstract of that data has been published; "Inactivation of *P. falciparum* by riboflavin and light," L. Lippert, R. Watson, S. Doane, H. Reddy and R. Goodrich, Vox Sanguinis 2002; 83, S2:163; a copy is included at Appendix S. These preliminary encouraging results are being verified with a second assay system for parasite viability, incorporation of tritiated hypoxanthine. The effectiveness of RF will also be tested against a second organism, *Orientia tsutsugamushi,* the causative agent for scrub typhus.

Key Research Accomplishments

- Established an extensive manufacturing process improvement program, substituting USP reagents for non-USP materials;
- Collaborated with the Navy blood research effort in the development of a lipid encapsulated hemoglobin product;
- Established new insights into red blood cell preservation by developing a series of novel experimental additive solutions (EAS) and incrementally adjusting the concentrations of key components;
- Developed a novel experimental strategy for the examination of red cell preservation which greatly reduces the inter-donor variation and in turn the number of samples needed to measure statistically significant treatment differences;
- Established a laboratory for the measurement of autologous human red cell *in vivo* recovery and survival;
- Conducted evaluation of two red cell and two blood plasma pathogen inactivation processes which established their potential utility against two organisms of military significance;
- Contributed a substantial portion of the data which led to the Food and Drug Administration (FDA) clearance of a frozen red cell processing, extended storage system now being adopted by the military blood program; and
- Developed a frozen red blood cell process enhancement for the extension of the post deglycerolization shelf life.

Reportable Outcomes

- Operated and maintained a hemoglobin production facility which:
  - ➢ Manufactured 106.04 liters of diasprin cross-linked hemoglobin containing 11.32 kilograms of hemoglobin;
  - ➢ Manufactured three specialty hemoglobin products; and
  - ➢ Instituted manufacturing process improvements resulting in
    - ▪ Increased purity by 30%
    - ▪ Increased yield by 25%
    - ▪ Reduced the time to produce a batch of hemoglobin solution
    - ▪ Publication of a manuscript in a peer-reviewed journal

- Operated and maintained a red cell storage laboratory which completed a series of interrelated *in vitro* research protocols and *in vivo* red cell survival and recovery clinical trials which resulted in data supporting:
  - Extension of the shelf life of refrigerated red blood cells for the current six weeks to twelve weeks;
  - The award of a patent for storage solutions extending the shelf life of refrigerated red cells for transfusion;
  - Eleven full-length articles published in peer-reviewed journals;
  - Publication of eight abstracts; a ninth has been accepted for presentation at a national scientific conference; and
  - Six masters of sciences research theses, seventh student's project is in progress and approximately 50% completed.

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Conclusions

The hemoglobin production facility (HPF) manufactured a large quantity, 106.04 liters, of diasprin cross-linked hemoglobin solution for use by the government. The hemoglobin solution was of high purity and carefully characterized. The manufacturing process was refined and the details and results of those refinements published in the peer-reviewed literature. At the direction of the government, the HPF was prepared for indefinite shut down.

The red cell storage research laboratory produced data resulting in the development of additive solutions, which extended the refrigerated shelf life of red blood cells from the six to twelve weeks. The further development and eventual use of this technology awaits recruitment of a commercial partner to manufacture the products and pursue clearance by the Food and Drug Administration. The work done under this contract by The Bionetics Corporation advanced the WRAIR Blood Research mission.

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Appendix A - (An improved process for the production of sterile modified hemoglobin solutions)

Biologicals (1997) **25**, 257–268

An Improved Process for the Production of Sterile Modified Haemoglobin Solutions

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Abstract. The process for manufacturing bulk quantities of sterile solutions of human haemoglobin (Hb) cross-linked between the alpha chains (ααHb) with bis(3,5-dibromosalicyl) fumarate (DBBF) was modified to: (1) improve product purity; (2) increase product yield; (3) eliminate non-United States Pharmacopoeia materials; (4) reduce reagent costs; and (5) reduce production time. These process modifications were the result of increased scientific understanding of the Hb cross-linking chemistry and were in the form of engineering and procedure controls that reflect current good manufacturing practices (cGMP). Purity, as reflected in the fractional yield of the desired ααHb product, has increased from 60% to 90+% of total Hb, and uncross-linked Hb was virtually eliminated. Impurities such as pyrogens, methaemoglobin, phospholipid, and free iron were reduced. The net yield of ααHb was increased from 33% to 58% of starting Hb content. Production time, the use of overtime, the consumption of expensive reagents and filters, and losses because of contamination have all been reduced. As a result, cost per gram of ααHb produced has decreased 60%. With this improved process, efficient production of very pure ααHb is possible.

Introduction

The U.S. Army produces kilogram quantities of modified haemoglobin from outdated human red blood cells to support research in the development of red blood cell substitutes. The Army seeks a blood substitute to serve as a universal oxygen-carrying resuscitation fluid on the battlefield. Haemoglobin, with its high oxygen carrying capacity, moderate colloid osmotic activity, and low antigenicity, is the most likely candidate blood substitute material.[1] Volumes greater than 100 litres per year of sterile 10 g/dl haemoglobin solutions are required to support this ongoing research and development process. The total volume is large because the safety and efficacy testing of resuscitation fluids require the infusion of substantial volumes of the material. Further, the manufacture of a major derivative product, a liposome-encapsulated haemoglobin, is only 15% efficient and requires large volumes of starting material.[2] Additional volumes of the haemoglobin are used for research into several types of brain injury and for basic studies of haemoglobin function and modification.[3,4]

The Army's original haemoglobin production facility was built at the Letterman Army Institute of Research (LAIR) on the Presidio of San Francisco in 1990. Descriptions of the original process and product have been published.[5] In 1993, with the closure of the Letterman Institute, the facility was moved to the Walter Reed Army Institute of Research (WRAIR) and re-established. The facility was rebuilt by a civilian engineering firm, brought to operation by Army personnel, and then turned over to an industrial operating company for continued operation and maintainance.

To understand the rationale for the process of haemoglobin recovery, modification, and purification, a brief discussion of the biochemistry and physiology of haemoglobin is necessary. Haemo-

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globin is the primary oxygen carrier in all higher animals. Normally, it is present in red blood cells, which provide the environment necessary for its proper function and protect the haemoglobin from degradation. Haemoglobin is a classic allosteric protein in that a ligand or effector molecule binding at one site on the haemoglobin can change the binding of ligands at other sites.[6] When the haemoglobin molecule binds oxygen, it changes conformation. Low affinity deoxyhaemoglobin, with four potential oxygen binding sites, turns into higher affinity partially liganded oxyhaemoglobin. In this way, oxygen affinity increases with each subsequent oxygen molecule bound, an effect called co-operativity. This co-operativity is delicately balanced to allow blood to saturate with oxygen in the lung and yet unload the oxygen as oxygen partial pressures decline in the tissues. Co-operativity is usually visualized as the sigmoid curve that relates oxygen partial pressure to the fractional saturation of haemoglobin. It is often expressed as a logarithmic function of the slope of the curve (the Hill number) at the point where the haemoglobin is 50% saturated (the P_{50}).[7]

In the red blood cell, haemoglobin oxygen affinity is regulated by H^+, CO_2, Cl^-, and 2,3-diphosphoglycerate (2,3-DPG). 2,3-DPG is the most important of these effectors in normal cells, stabilizing the deoxy-form and reducing the oxygen affinity. When haemoglobin is removed from red blood cells, most of the 2,3-DPG and CO_2 disperse. Without these effectors, the oxygen affinity rises as seen in the decrease in the P_{50} from 27 Torr to 10 Torr. Cooling further increases the affinity of haemoglobin stripped of effectors from a P_{50} of 10 Torr at 37°C to 4 Torr at 5°C.[8] The unloading of oxygen then becomes difficult because of the small partial pressure gradient available to drive it away.

Structurally, native haemoglobin is a tetramer composed of two alpha globin and two beta globin chains ($\alpha\alpha\beta\beta$). Each individual globin protein chain is folded to surround an iron-containing haem group where oxygen is bound and released. The folding of the monomers and their assembly into a tetramer are all controlled by non-covalent interactions. These interactions are weakest between the like globin chains, so haemoglobin tetramer is in rapid equilibrium with the low concentrations of heterodimer [$\alpha\alpha\beta\beta \Leftrightarrow 2(\alpha\beta)$][9,10] [Fig. 1(a)]. Within the red blood cell, heterodimers are retained and rapidly reassociate back to tetramer. Outside the red blood cell, the free heterodimer is not desirable because it is filtered and accumulated by the kidney, causing renal damage. Even the unmodified tetramer is also not desirable in the circulation because it dissociates into heterodimers and because unmodified tetramer binds oxygen more avidly and less co-operatively than normal blood.

In manufacturing a haemoglobin-based blood substitute, the problems of tetramer–dimer equilibrium and high oxygen affinity are addressed by modifying the haemoglobin tetramer. The haemoglobin modification used in this process, cross-linking the α-globin chains from one α-lysine-99 to the other with fumarate,[11] prevents the formation of the dimer and subsequent renal damage [Fig. 1(b)]. In addition, cross-linking between the α-lysine-99s makes the binding and release of oxygen by the cross-linked tetramer ($\alpha\alpha$Hb) much like that of normal blood.

Another concern of manufacturing is maintaining the integrity of the product. Despite the relative stability of haemoglobin, heat, shear, and oxidative stress can all damage the molecule. Oxidation of Fe^{2+} haemoglobin to Fe^{3+} methaemoglobin is the usual first step in the degradation of oxygenated solutions at room or body temperatures. Inside the red blood cell haemoglobin oxidation is quickly reversed by the methaemoglobin reductase enzyme system. Outside the red blood cell the conversion to methaemoglobin proceeds unchecked. The molecule can further degrade with the loss of haem and iron and the unraveling of the protein to form linear strands that aggregate and precipitate. The haemoglobin breakdown products, haem and iron, can potentiate oxygen free radical reactions and lead to further protein damage.[12]

With the structural and functional characteristics of haemoglobin in mind, the facility and procedures developed at LAIR were modified to increase the yield and purity of the product and the efficiency and reproducibility of the process. This paper describes the new production process, the improvements in the quality of the product, and reviews the critical changes. This information is presented as a model for the large scale production of modified haemoglobin.

The new modified haemoglobin production process

Process overview

The production of a 20-litre sterile batch of $\alpha\alpha$Hb solution requires 30 working hours over a five-day

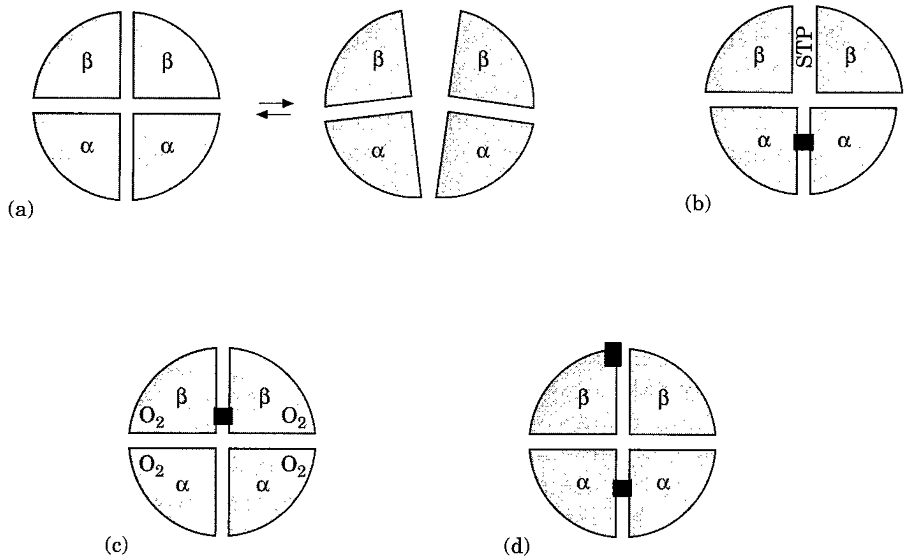

Figure 1. Schematic of Haemoglobin and its modification. (a) Haemoglobin is a tetrameric globular protein composed of two alpha (α) and two beta (β) chains. The chains are not covalently held together. At high concentrations, the tetramer dominates while at low concentrations the heterodimer is favoured. (b) There are two sites where the compound bis(3,5-dibromosalicyl) fumarate (DBBF) can covalently cross-link (shown as ■), β-lysine-82 to β-lysine-82 and α-lysine-99 to α-lysine-99. Deoxygenated haemoglobin favours cross linking between the two α-lysine-99s. In addition, the β-lysine-82 sites can be blocked with sodium tripolyphosphate (STP), optimizing the desired product $\alpha\alpha$-cross-linked haemoglobin. (c) Oxygenated haemoglobin will primarily cross link between the two β-lysine-82s, forming $\beta\beta$-cross-linked haemoglobin. (d) Extra DBBF can react with other lysine sites such as a univalent linkage to the β-chain terminal amino group (β-NH_2).

period. All processes are conducted at 5°C unless otherwise specified. On the first day, 80 units of outdated human red blood cells (RBCs) are pooled into a sterile vessel. On the second day, the RBCs are washed by cross-flow filtration with an isotonic saline solution and lysed with a hypotonic phosphate solution. The resulting haemolysate solution, which starts with 97·5% of its protein content as haemoglobin, is purified by filtration through 0·1 μm and 500 kDa filters, eliminating the stromal material. Haemoglobin is then concentrated with a 5 kDa filter, removing low molecular weight impurities with the permeate. Sodium tripolyphosphate (STP) is added to the haemoglobin to increase the buffering capacity of the solution and to block competing cross-linking sites at the β-lysine-82s and β-NH_2 in the $\beta_1\beta_2$ cleft [Figs 1(c) and 1(d)]. The solution is then transferred to a bioreactor. On the third day, the stroma-free haemoglobin solution in the STP "blocking" buffer is warmed to 25°C, the solution is deoxygenated, and the haemoglobin is cross-linked with bis(3,5-dibromosalicyl) fumarate (DBBF). The cross-linked haemoglobin solution is then heat treated at 76°C and pH 6·9 for 90 min to denature selectively unmodified haemoglobin and inactivate some potential viral contaminants. On the fourth day, the contents of the bioreactor, including about 750 g of denatured protein, are filtered, oxygenated, refiltered through the 0·1 μm and 500 kDa filters, and formulated in the final physiological buffer by cross-flow diafiltration using the 5 kDa filter. On the morning of the fifth day, the resulting solution is sterile packaged and frozen at −80°C. More than 70 standard operating procedures (SOPs) support facility operations by detailing facility cleaning, buffer preparation, in-process analysis, and other quality control procedures.

First day procedures

On the first day of the production process (Fig. 2), 80 units of outdated human red blood cells, not more than 63 days from the date of donation, are pooled. Pooling is accomplished by transfer from the original bags into a 30-litre sterile tank T0 (Alloy Products Corp., Waukesha, WI) via

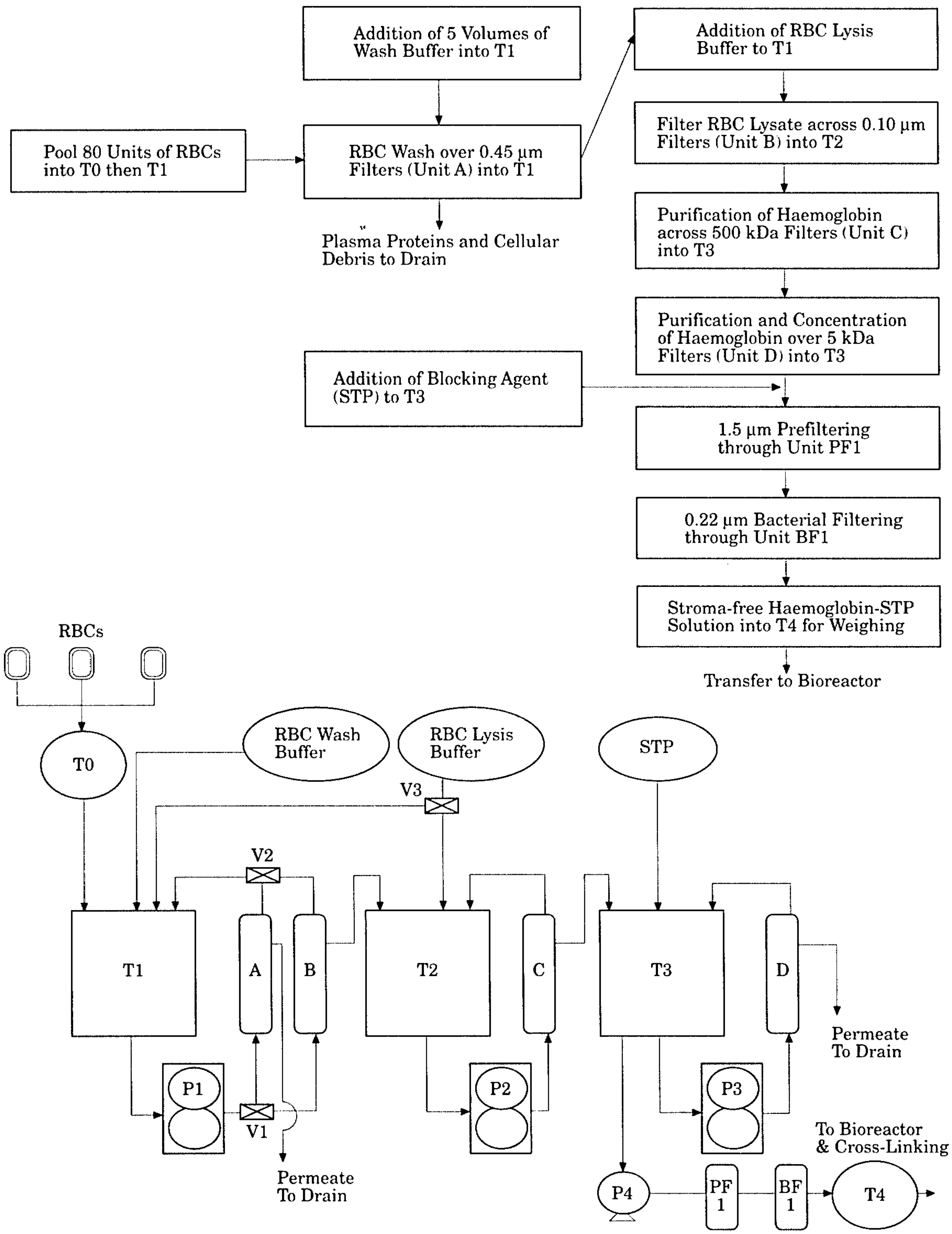

Figure 2. Process and flow diagrams for days 1 & 2. Red blood cells are pooled, washed, and lysed. Cell fragments are removed by stepwise filtration, generating stroma-free haemoglobin solution.

clinical blood administration sets with 170–260 μm nylon aggregate filters (IC8087, Baxter Healthcare Corp., IV Division, Deerfield, IL). Experience has shown that clinically incompatible blood types can be mixed and stored overnight at 5°C without lysis.

Second day procedures

On the second day (Fig. 2), 20 litres of 0·9% W/V sodium chloride (isotonic saline) is added to the 100-litre tank T1 (DCI Inc., St Cloud, MN) to prime the 0·45 μm filter bank A (CFP4-E-55 SMO, A/G Technology Corp., Needham, MA). The priming volume is circulated by pump P1 (Model 60, Waukesha Fluid Handling, Waukesha, WI) in the closed circuit consisting of valve V1, 0·45 μm filter bank A, valve V2 and tank T1. The pooled cells (~20 litres) are then added to the 20-litre priming volume in T1. The cells are washed with isotonic saline, and the plasma proteins are removed with the permeate of filter bank A. A constant volume of 40 litres is maintained with the addition of isotonic saline into T1. Washing of the red blood cells is complete when no albumin is spectrophotometrically detected at a wavelength of 280 nm in the permeate of filter bank A. Two-hundred litres of isotonic saline is sufficient to accomplish the RBC washing.

Washed cells are then lysed by adding hypotonic 10 mM phosphate buffer at pH 7·60 (lysis buffer) through valve V3 into tank T1 while maintaining the constant 40-litre volume. Valves V1 and V2 are then switched to bypass filter bank A, and the lysed cells are pumped with pump P1 through valve V1, the 0·1 μm filter bank B (CFP-1-E-55SMO, A/G Technology Corp., Needham, MA), valve V2, and back into tank T1. The haemoglobin contained in the permeate from filter B flows into the 100-litre tank T2 (DCI Inc., St Cloud, MN) while the retentate containing the RBC membranes flows back to tank T1. Lysing continues until $\leq 0·5$ g/dl haemoglobin is detected[13] in tank T1. Upon completion of lysing, valve V3 is switched and lysis buffer is added directly to tank T2 to sustain a minimum circulation volume. Purification of the haemoglobin continues by circulation through pump P2 (Model 30, Waukesha Fluid Handling, Waukesha, WI) through the 500 kDa filter bank C (UFP-500-E-55SMO, A/G Technology Corp., Needham, MA) and back into tank T2. The haemoglobin contained in the permeate from filter bank C flows into the 40-litre tank T3 (DCI Inc., St Cloud, MN) and the retentate containing high molecular weight (>500 kDa) impurities returns to tank T2. Further purification and concentration occur by pumping the solution in tank T3 with pump P3 (Model 60, Waukesha Fluid Handling, Waukesha, WI) through the 5 kDa filter bank D (UFP-5-E-55SMO, A/G Technology Corp., Needham, MA) and back into tank T3. Molecules <5 kDa (salts, buffers, water, etc.) are discarded in the permeate of filter bank D and the haemoglobin is retained in tank T3.

The solution is concentrated to 10 g/dl of haemoglobin and weighed in T3. A solution of 20 mM STP at pH 6·50 is added to the haemoglobin in tank T3 in sufficient volume to produce an 8:1 molar ratio of STP to haemoglobin. The resulting solution is pumped with pump P4 through a 1·5 μm prefilter PF1 (ZCPP1-1·5C, Domnick Hunter, Inc., Charlotte, NC) and 0·2 μm bacterial filter BF1 (ZCMA1-020C-P, Domnick Hunter, Inc., Charlotte, NC) into weighing tank T4 (Alloy Products Corp., Waukesha, WI). Finally, the solution is weighed, transferred to the bioreactor, cooled to 5°C, and stirred at 200 rpm overnight.

Third day procedures

Day three of the production process (Fig. 3) begins by warming the haemoglobin solution in the bioreactor BR (LSL Biolafitte, France) to 25°C to facilitate rapid deoxygenation.[8] The headspace of BR is purged with nitrogen and the solution is deoxygenated by pumping it with pump P4 (701S, Watson-Marlow, Concord, MA) through two membrane oxygenators MO1 & MO2 (Univox, Baxter Healthcare Corp., Bentley Division, Irvine, CA) connected in series and back into the BR. Nitrogen gas flows countercurrent to the haemoglobin through MO1 and MO2 (gas to solute flow rate of 1:1) at 7 litres per min. Oxygen removal is monitored spectrophotometrically (Model 8452A, Hewlett Packard, Palo Alto, CA) at 758 nm by means of the optical flow cell FC (Custom Sensors & Technology, St Louis, MO). When the spectrophotometer indicates that >95% of the oxygen bound to the haemoglobin has been removed, deoxygenation is discontinued. The haemoglobin solution is then heated to 37°C. The cross-linking reagent, DBBF, is added to the haemoglobin in 1·9:1 molar ratio as a slurry of the dry powder in 2 litres of pyrogen free water. The cross-linking reaction is allowed to proceed for 3 h. Immediately following cross-linking, the pH is adjusted to 6·90 with 5 M NaOH and the solution is warmed to 76°C to inactivate viruses and selectively denature unmodified haemoglobin. After 90 min, the solution is cooled to 5°C and stored overnight under nitrogen.

Fourth day procedures

On the fourth day (Fig. 4), the contents of the bioreactor, including at least half a kilogram of suspended denatured haemoglobin and precipitated

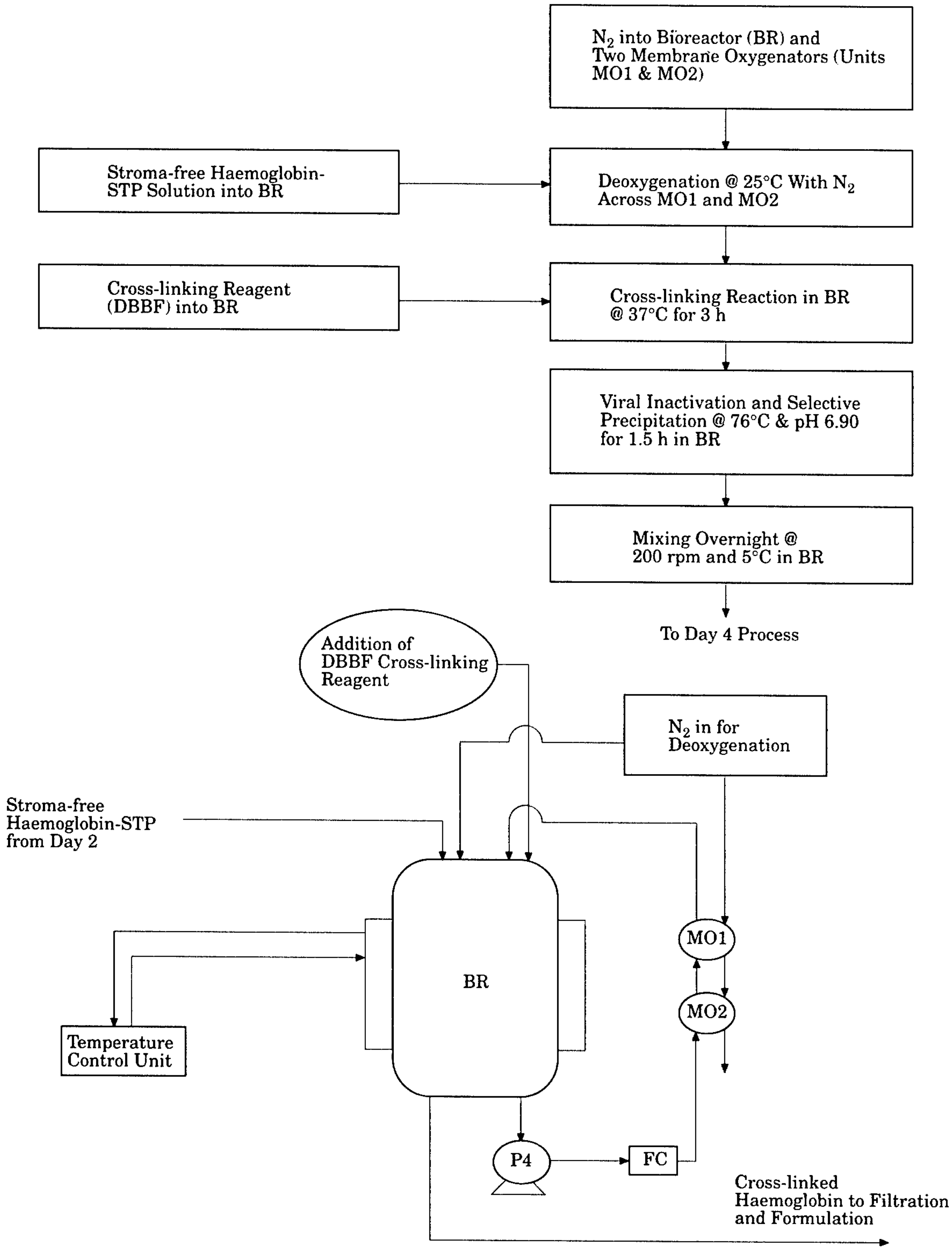

Figure 3. Process and flow diagrams for day 3. Stroma-free haemoglobin is cross-linked with DBBF, yielding $\alpha\alpha$Hb. Heat treatment accomplishes both pasteurization and purification of the cross-linked product.

haemoglobin sludge, are filtered through a 70 µm prefilter PF2 (RM3F700H21, Pall Corp., East Hills, NY), a 10 to 3 µm depth filter DF (5580502W3, Sartorius Corp., Bohemia, NY), and collected in transfer tank T5 (Alloy Products Corp., Waukesha, WI). Haemoglobin from the holdup volumes of

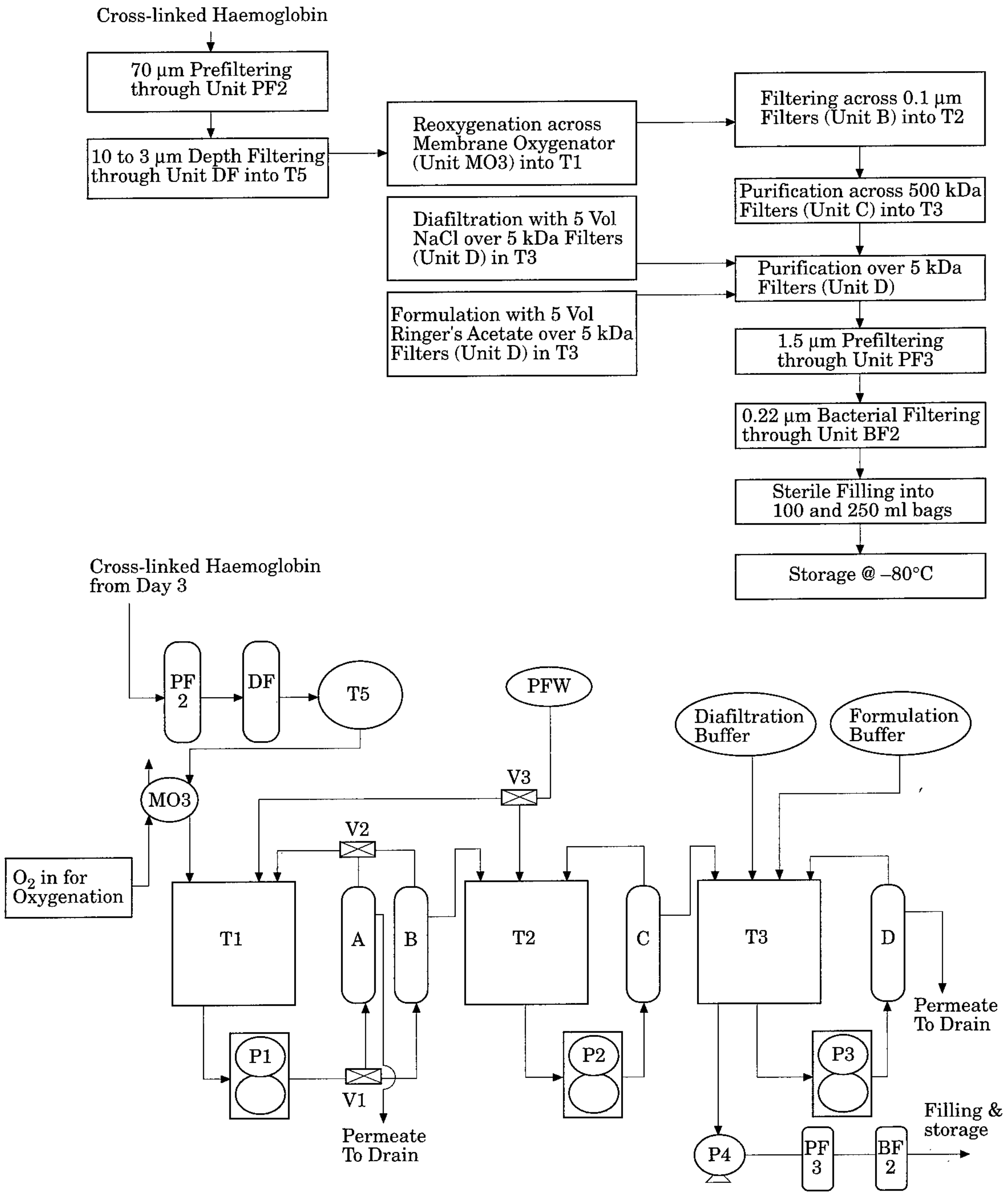

Figure 4. Process and flow diagrams for day 4. ααHb is filtered stepwise through the cross flow filtration system, removing particulate matter and producing the final ααHb product.

the filters is recovered by rinsing with 60 litres of isotonic saline. The haemoglobin solution is reoxygenated by pumping countercurrent to oxygen flow through membrane oxygenator MO3 (Univox, Baxter Healthcare Corp., Bentley Division, Irvine, CA) and into tank T1. Purification is accomplished by repeating the cross-flow filtration from day two in the cascading pumped loops formed by tank T1 and filter bank B, tank T2 and filter bank C, and tank T3 and filter bank D. Twenty litres of pyrogen-free water is added to each of tanks T1 & T2 at the end of their respective portions of the filtration to maximize haemoglobin recovery from the holdup volume. Phosphate and other low molecular weight solutes are removed in the permeate of filter bank D with 200 litres of isotonic saline added to tank T3.

Final formulation is accomplished by concentrating the solution over filter bank D to 10 g/dl followed by cross-flow diafiltration with 140 litres of Ringers' acetate. The permeate from filter bank D of both buffers is discarded. The cross linked haemoglobin is then pumped with pump P4 through a 1·5 μm prefilter PF3 (ZCPP1-1·5C, Domnick Hunter, Inc:, Charlotte, NC), a 0·2 μm bacterial filter BF2 (ZCMA1-020C-P, Domnick Hunter, Inc., Charlotte, NC), and collected into a storage vessel where it is stored overnight at 5°C.

Fifth day procedures

On the fifth day, the haemoglobin is packaged. The storage vessel is brought from the refrigerator to a biological safety cabinet and connected to a sterile bag filling system composed of tubing and bags, a variable-flow peristaltic pump, a digital scale, haemostats, bag seals, and crimpers. The ααHb is pumped into sterile, nonpyrogenic biological storage bags (RCM-93A-4-MLL, Stericon Inc., Broadview, IL) in 100 ml and 250 ml quantities, sealed, and labelled. The ααHb bags are placed in bubble-wrap envelopes, boxed, and frozen at –80°C.

Quality control and management

A Haemoglobin Production Committee, the authors, directed the changes in the production process and agreed to use current good manufacturing practice (cGMP) as their goal. More than 70 standard operating procedures (SOPs) were written including eight defining requirements for and the handling of source blood and other raw materials, 19 describing equipment operation and maintenance, 14 detailing production procedures, seven describing cleaning procedures, eight specifying documentation and product inventory management and 21 detailing quality control laboratory procedures. Process control analytical chemistry and final analysis for batch release were done in a dedicated laboratory. Oxygen equilibrium curves, anion exchange chromatography, and rabbit pyrogen testing were performed in separate laboratories.

Results

The new process for producing modified haemoglobin yields four kinds of benefits. First, the total yields of modified haemoglobin produced in each production run has increased. Second, the specificity of the process to produce the desired product, human haemoglobin cross-linked correctly but not otherwise modified, has increased. Third, contaminants are reduced. Fourth, the cost of the process in regards to the value of materials added and the labour required has decreased.

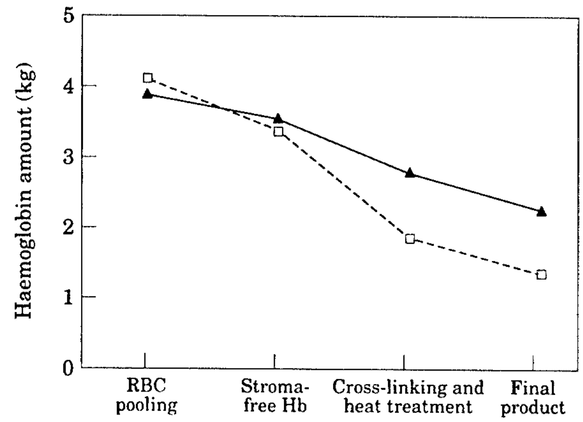

Figure 5. Haemoglobin production yields. Overall product yield has increased from 33% to 58%. Total haemoglobin amounts are measured after RBC pooling on day two at T0 (Fig. 2), on day two at T4 (Fig. 2) for stroma-free haemoglobin, on day four at T1 (Fig. 4) after cross-linking and heat treatment, and on day five at final filling and storage. (-□-), Letterman '91–92; (▲), Walter Reed '96.

Figure 5 shows the mass of haemoglobin at several points in the modified haemoglobin production process as performed at the Letterman Army Institute of Research (LAIR) in 1991–1992 and at the Walter Reed Army Institute of Research (WRAIR) in 1996. Yield as a fraction of the initial mass of haemoglobin has increased from 33 to 58%.

Figures 6(a) and 6(b) are anion-exchange fast performance liquid chromatograms of haemoglobin produced before and after the process changes. The dominant peak eluting at 4 min is the desired product, human haemoglobin cross-linked with fumarate between the α-lysine-99s but not otherwise modified. In Figure 6(a), the peak eluting at 3 min is uncross-linked haemoglobin. The peaks to the right of the dominant peak in both figures are haemoglobin cross-linked between the α-lysine-99s and further modified product with additional fumarate residues at the β-chain terminal amino group (β-NH_2), β-lysine-82, or cross-linking β-NH_2 and β-lysine-82 in the same β-chain. As can be seen, uncross-linked haemoglobin has been essentially eliminated and the proportion of the desired product has been increased from 50 to 90+%.

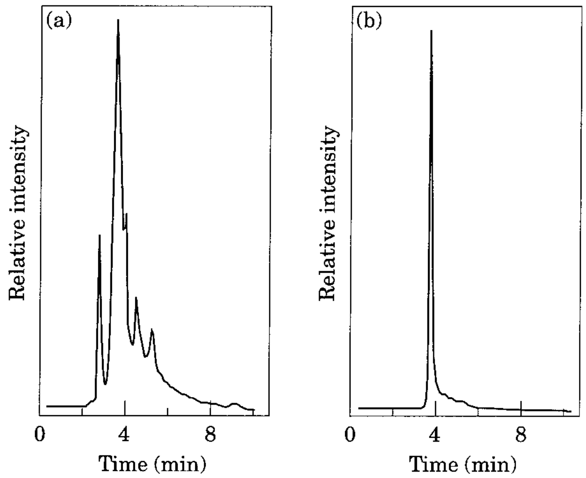

Figure 6. FPLC data on lot #950207 (prior to changes) and lot #960416. Analysis was carried out on a Pharmacia FPLC system using a mono Q HR 5/5 column at a flow rate of 1·0 ml/min. The absorbance of the eluent was measured at 405 nm with the Pharmacia on-line mercury lamp detection system with a 5 mm optical flow cell. The system was equilibrated with buffer A consisting of 20 mM Tris acetate at pH 8·3. The sample was loaded in a 100-µl volume and eluted with a gradient of 0–20% buffer B (20 mM Tris acetate, 1·0 M NaCl) over 20 min (or 1%/min). The major fraction, ααHb, elutes at 4 min.

Contamination of human-derived haemoglobin solutions can arise from the source blood, from materials purposely added in the haemoglobin purification and modification process, from microbiological growth, from materials leached from the walls of production vessels, and from the breakdown products of haemoglobin itself. Table 1 shows, that in comparing the old and new processes, the concentrations of organic phosphate from red blood cell membrane phospholipids, free iron from haemoglobin breakdown and tank walls, bacterial endotoxin and other pyrogens, and methaemoglobin are all either lower or remain acceptably low.

The cost of the process has also been reduced. Table 1 shows the savings in the use of pyrogen-free water. The elimination of HEPES buffer from the cross-linking step saves \$3000 in each production run. Implementation of engineering changes to allow fully in-place cleaning and sterilization of filters also saves about \$3000 per run. Reduced and restructured process time has eliminated the requirement for production crew overtime, which saves an additional several thousand dollars in each production run. The improvement in run yield and reduced loss of production runs because of pyrogens results in a 125% increase in useful product per run. This yield increase and the reduced costs mean that the cost per useful gram of modified haemoglobin produced is reduced by more than 60%.

Discussion

The goals of improving the modified haemoglobin production process were to increase purity and yield of the product and the efficiency and reproducibility of the process. These goals were translated into specific objectives that included: (1) reducing endotoxin and pyrogen contamination; (2) eliminating non-United States Pharmacopoeia (U.S.P.) materials; (3) reducing methaemoglobin

Table 1. Comparison of batches of cross-linked haemoglobin solution

Parameter (Units)	Old Process[5]	New Process
Haemoglobin (g/dl)	9·8	9·95
Methaemoglobin (%)	7·5	3·20
Total Yield (%)	33	58
pH	7·56	7·39
Sterility	Pass	Pass
Rabbit Pyrogen Test (% pass)	62·5	100
Total Phosphate (µg/ml)	<1	0·75
Free Iron (µg/ml)	4·2	4·57
Pyrogen free water used (litres)	1897	1197
Total labour (h)	180	132

Comparative analytical results obtained for haemoglobin production runs acquired at Letterman Army Institute of Research (old process) and at Walter Reed Army Institute of Research (new process). Data for old process is taken from either reference 5 or is the average value from at least three production runs. Data for the new process is the average of four production runs.

formation; (4) reducing the amount of uncross-linked haemoglobin formed; (5) reducing the formation of $\alpha\alpha$-cross-linked haemoglobin that was then further modified; (6) minimizing the in-process denaturation of haemoglobin; (7) recovering haemoglobin previously lost in holdup volumes; (8) speeding red blood cell pooling and washing; (9) speeding deoxygenation and oxygenation; (10) reducing the use of expensive ingredients; (11) reducing overall production time; and (12) elimination of routine requirements for over-time. Because of the potentially conflicting nature of the objectives, and perhaps even the goals, an organization for the management of process improvement was established and a commitment to current good manufacturing procedures was made.

Review of records from production runs at Letterman in 1991 and 1992 led to the recognition that 37% of runs were lost because of contamination by pyrogens, and the most significant physical loss of the product was in the holdup volumes of tanks and tubing. Engineering solutions to these problems included the application of full sterilize-in-place capability for the cross-flow filtration system and alterations in filtration and holdup volumes. Laboratory quality control assured reproducible equipment sterility and no increase in haemoglobin denaturation with reduced fluid volumes.

Other product purity issues were addressed in the alteration of the red blood cell washing process, attention to factors reducing methaemo-globin formation, elimination of HEPES buffer in the cross-linking process, and better handling of the cross-linking reagent. The changes reflect scientific, engineering, quality management, and economic decisions and are discussed separately below.

Outdated units of RBCs are now pooled into a sterile holding tank on the first day. The separation of the pooling step allows the production of stroma-free haemoglobin to take place within a single 8-hour work shift on the second day. Pooling clinically incompatible red blood cells does not lead to agglutination or lysis or to increased concen-trations of red blood cell phospholipids in the final product. Opening the RBC storage bags a day earlier caused no detectable increase in bacterial or endotoxin contamination.

To avoid methaemoglobin formation, the haemo-globin modification process takes advantage of the thermodynamic stability afforded by low temperature, pH control, and deoxygenation. Engin-eering changes now keep the process at 5°C except for deoxygenation, cross-linking, and heat treat-ment. Low pH is avoided and the heat denaturation process is carried out at pH 6·9. Deoxygenation is critical for two reasons. First, it forces the haemo-globin to be in the proper configuration for cross linking between the α-lysine-99s[14] and second, it prevents oxidation during the high temperature denaturation of impurities at the end of cross-linking.[15] The speed of deoxygenation and reoxy-genation are important because the methaemo-globin produced is enhanced when haemoglobin is in the partially oxygenated state.[16] Faster conver-sion from oxyhaemoglobin to deoxyhaemoglobin and back reduces the presence of unstable partially oxygenated haemoglobin. A single pass through one gas exchange membrane, a clinical membrane oxygenator with nitrogen as the gas medium, fails in removing all of the oxygen from the solution. Continual cycling through the gas exchange membrane is required for complete deoxygenation. The time required for oxygen removal has been reduced from 3 to 1·5 h with two membrane oxygenators in series. For reoxygenation, a single pass through one gas exchange membrane with oxygen as the gas medium results in complete oxygenation, eliminating the requirement for dilution into 400 litres of oxygenated buffer. Along with the new deoxygenation and oxygenation procedures, success in gently handling the molecule has resulted in only 2–3% methaemoglobin in the final product.

In published descriptions of the cross-linking of haemoglobin with DBBF, HEPES buffer was used for both its buffering capability and as a solvation medium for DBBF. However, HEPES is not a U.S.P. listed product, and its presence raises questions about its toxicity. Haemoglobin itself as well as the STP "blocking" agent are adequate buffers at the pH used (7·0) and the solvation of DBBF in HEPES prior to exposure to a haemoglobin solution is not required for the cross linking reaction.[14] Therefore, HEPES buffer has been removed from the manufac-turing process.

The chemical purity of the $\alpha\alpha$-cross-linked haemoglobin product, uncontaminated by uncross-linked haemoglobin or by further modified cross-linked haemoglobin, is related to the ability to deliver the intact cross-linking agent in the correct stoichiometry under the correct conditions. To understand the optimization of the cross-linking of haemoglobin, a description of the reaction of DBBF with haemoglobin is necessary.

The intramolecular cross-linking of haemoglobin is accomplished by a nucleophilic displacement reaction involving the cross-linker, DBBF, and the side-chain of lysine. DBBF is a diester with very efficient leaving groups in the dibromo-aspirins. The side-chain amine group of lysine with its lone pair of electrons attacks one of the carbonyl groups of fumarate. The dibromo-aspirin leaves, and an amide bond is formed with the fumarate. The same nucleophilic displacement reaction occurs at the opposite end of the DBBF, yielding a final product of intramolecularly cross-linked haemoglobin.

Several factors contribute to the specificity of the cross linking between DBBF and deoxyhaemoglobin. Solution conditions, protein conformation, allosteric effectors, and alternate site blockers all help to increase the yield of the cross-linking reaction.[14] In addition, specific interactions between DBBF analogues and haemoglobin have been observed by X-ray crystallography that position the DBBF optimally for cross linking at the α-lysine-99 site.[17]

Of the 44 lysines in haemoglobin that potentially react with DBBF, only a few are active. In analysing the final product, only α-lysine-99, β-NH_2 and β-lysine-82 react substantially with DBBF.[18] The major reason for this specificity is probably the high value of the pK_a of the lysine side-chain amine. With a pK_a of 10·8 in aqueous solution, free lysine is highly protonated under neutral conditions. Since the reaction conditions are maintained at pH 6·9, lysines exposed to the aqueous medium are largely protonated. With a proton associated with the lone pair of electrons, lysine cannot attack the fumarate carbonyl groups of DBBF. Thus, lysines that are on the periphery of the haemoglobin molecule are unreactive toward the DBBF. Lysines buried inside the haemoglobin are simply inaccessible to the DBBF. Only α-lysine-99, β-NH_2 and β-lysine-82, all in the central cavity of haemoglobin, are accessible. The increased hydrophobic environment of the cavity favours the neutral form of lysine and effectively lowers the pK_a of these lysines. With the proton now removed, these lysines can effectively react with the DBBF. The use of a β-cleft blocker (STP) effectively blocks the β-NH_2 and the β-lysine-82 sites. Acidic conditions (pH < 7), though potentially causing increased oxidation, must be used in order for the STP to bind efficiently within the beta cleft.

As mentioned previously, chloride and CO_2 act as allosteric effectors of haemoglobin. The chloride interaction involves several binding sites that include α-lysine-99. This reaction elevates the population of protonated lysines, rendering the amino acid inactive for the cross-linking reaction. Therefore, chloride is removed from the reaction mixture.

The cross-linking reaction between α-lysine-99s only occurs with haemoglobin that is not carrying oxygen. If oxygen is present, the haemoglobin changes conformation to its higher affinity form. In this form, the α-lysine-99s are no longer available and a different reaction occurs cross-linking the β-chains between the β-lysine-82s. This creates a modified haemoglobin that gives up oxygen less readily.[19] Because of this competing reaction, the desired reaction is performed with the solution fully deoxygenated and with a blocking compound occupying the alternative cross-linking site in the space between the β-chains.

Finally, the cross-linking agent, DBBF, is unstable in water. It is susceptible to hydrolysis that can be catalysed both by acidic or basic conditions. Since DBBF has two reactive sites, hydrolysis at one reactive site of the DBBF renders it incapable of intramolecular cross-linking. However, the second unhydrolysed reactive site of DBBF is still capable of reacting with lysine sites on the haemoglobin. Prevention of DBBF hydrolysis becomes a high priority to ensure intramolecular cross-linking and deter reactions with partially hydrolysed DBBF. As conjugate bases and acids hydrolyse DBBF, DBBF is rapidly added to deionized pyrogen-free water, and the slurry is then immediately transferred to the bioreactor to minimize hydrolysis of the cross-linking reagent. Deoxygenation of the slurry is not performed because the oxygen dissolved in the pyrogen free water of the slurry, about 9 ml, would only oxygenate 0·2% of the haemoglobin and thus does not affect the process yeld. In fact, the small amount of oxygen present may account for the substantial decrease in the unmodified product. Unmodified haemoglobin has a higher affinity for oxygen than $\alpha\alpha$-cross-linked product and is more likely to bind oxygen. Oxygenated haemoglobin denatures more rapidly than deoxygenated haemoglobin.[15,20] Therefore, the small amounts of oxygen may enhance the denaturation of undesired material. The homogeneity of the cross-linked product can be ascertained with anion exchange FPLC [see Figs 6(a) and 6(b)]. By preserving the integrity of the DBBF, synthesis of pure $\alpha\alpha$Hb as measured by FPLC has increased from 50 to 90+% with a significant increase in overall product yield.

Better understanding of the cross-linking reaction chemistry and the implementation of current good manufacturing practices have allowed substantial increases in the purity and yield of the modified hemoglobin product. The high specificity of this reaction and the potential for further refinement was not realized until the process changes were undertaken. Making very precise chemical modifications of a globular protein is possible.

This increased understanding and control of the reaction chemistry and the production process has led to savings of time and money. Saving time feeds back into product quality by reducing the exposure of hemoglobin to denaturing environments. Saving money occurs through reduced raw material, process, and labour costs and a reduced potential for mishaps. Freed resources can be used in other parts of the development program.

This report documents the recent progress of work originally begun by Winslow and Chapman[5] to produce a model modified hemoglobin solution of high purity to advance the development of oxygen-carrying resuscitation fluids. It shows that current good manufacturing practice production of biological material can be carried out effectively and efficiently in the public sector. It shows the potential for very specific and high yield chemical modification of hemoglobin.

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Appendix B - (A hypotonic storage solution did not prolong the viability of red blood cells)

A hypotonic storage solution did not prolong the viability of RBCs

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BACKGROUND: Hypotonic storage solutions and WBC filtration are both reported to improve RBC viability. This study tested the ability of an investigational hypotonic storage solution (AS-24, Medsep Corp.) to extend the viability of liquid-stored RBCs to 8 weeks.

STUDY DESIGN AND METHODS: In a pair of crossover trials, 11 RBC units, WBC-reduced by filtration and stored in AS-24 for 8 weeks, were compared with units from the same donors that were stored for 6 weeks in AS-3, and 13 RBC units, WBC-reduced by filtration and stored in AS-3 for 8 weeks, were compared with units from the same donors that were stored for 6 weeks in AS-3. Viability was measured by the $^{51}Cr/^{99m}Tc$ double-isotope method.

RESULTS: RBC viability at 8 weeks averaged 64 ± 3 percent in the AS-24 units and 67 ± 2 percent in the AS-3 units. It was equal at 77 ± 3 percent and 77 ± 2 percent after 6 weeks' storage in AS-3 in both trials.

CONCLUSIONS: Prestorage WBC reduction and storage in AS-24 did not extend RBC viability to 8 weeks. The improved viability previously demonstrated with storage of dilute suspensions of RBCs in hypotonic solutions is probably caused by factors other than the hypotonicity.

ABBREVIATIONS: MCV = mean corpuscular volume; RT = room temperature.

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Extending the shelf life of liquid-stored RBCs from 6 weeks to 8 weeks has the potential to increase the availability of blood in remote areas and improve the utility of autologous blood storage for elective surgery.[1] Ideally, an RBC component with an extended shelf life should be physiologically compatible with transfusion in large volumes, should retain conventional values of storage Hct and volume, and should conform to standard manufacturing practices.

In 1986, Meryman et al.[2] suggested that increasing the membrane surface tension of stored RBCs would limit membrane loss by reducing the budding of microvesicles from surface spicules that develop on preserved cells. They reported that hypotonic solutions that induced RBC swelling in vitro could be used to store RBCs for more than 14 weeks with greater than 75 percent viability. Additional reports have corroborated this finding.[3-9] However, those reports have two serious limitations. First, none of the studies examined the effect of hypotonic solutions alone in a system with a standard volume, a conventional Hct, or salts compatible with transfusion. Second, most of these studies relied on surrogates for viability as endpoints, such as RBC morphology and RBC ATP concentration.

The two studies reported here were undertaken to determine the ability of a hypotonic storage solution (AS-24, Medsep Corp., Covina, CA) to extend the viability of RBCs to 8 weeks. AS-24 is a hypotonic solution of the type described by Meryman et al.[2] and was manufactured under a license of their patent. The ability to extend viability was measured directly in a randomized crossover study comparing the viability of RBCs stored in AS-24 for 8 weeks with that of RBCs from the same donors that were stored in an isotonic commercial AS (AS-3, Nutricel, Medsep Corp.) for 6 weeks. In a parallel study, the viability of RBCs stored in AS-3 for 8 weeks was compared with that of RBCs from the same donors that were stored in AS-3 for 6 weeks. In each crossover study, the RBCs in the 8-week arm were WBC-reduced by filtration in an attempt to further improve viability,[10] whereas the RBCs in the 6-week arm were not WBC-reduced, so as to provide a conventionally stored standard. Viability was measured by the $^{51}Cr/^{99m}Tc$ double-isotope

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technique 24 hours after the donor received, by IV infusion, a labeled sample from the RBC unit.

MATERIALS AND METHODS

Volunteers

Thirty healthy volunteers meeting standard blood donor criteria[11] were enrolled in one study or both studies after giving informed consent in accordance with protocols approved by the Institutional Review Board of the Walter Reed Army Institute of Research and the US Army's Human Subjects Research Review Board. There were 30 subjects in all, with 2 subjects participating in both studies. Volunteers were tested to exclude sickle Hb trait (Sicklescreen, Pacific Hemostasis, Huntersville, NC) and abnormal osmotic fragility (Unopette Test 5830, Becton Dickinson, Rutherford, NJ).

Storage solutions

The compositions of AS-3 and AS-24 are shown in Table 1. The significant differences in the solutions are the presence of mannitol and the absence of sodium chloride in the AS-24 and vice versa in the AS-3. Both solutions were formulated to have a pH of 5.8 and were used as 100-mL ASs for packed RBCs collected into CP2D.

Study design

We conducted two parallel, randomized, crossover studies. Each study allowed the direct comparison of the viability of RBCs stored for 8 weeks and that of cells from the same donor stored for 6 weeks in a standard, licensed AS. The results of the two studies were then compared. In the first crossover study, RBC units were WBC-reduced by filtration and stored in AS-24 for 8 weeks. They were compared with non-WBC-reduced units from the same donors that were stored for 6 weeks in AS-3. In a second trial, RBC units were WBC-reduced by filtration and stored in AS-3 for 8 weeks. They were compared with second units from the same donor that were stored for 6 weeks in AS-3. Electrolytes, pH, glucose, lactate, RBC morphology scores, and RBC ATP concentrations were measured at the beginning and the end of storage. Viability was measured by the $^{51}Cr/^{99m}Tc$ double-isotope method.[12] Postinfusion survival of the RBCs was measured as the RBC ^{51}Cr retention, corrected for radioactive decay and 1 percent per day elution.

TABLE 1. Components of AS-24 and AS-3 in m*M* and solution pH

	AS-24	AS-3
Adenine	2	2
Dextrose	68.8	55.5
Mannitol	13.8	0
Na_3 citrate	17.7	20
NaH_2PO_4	13	23
NaCl	0	70.1
H_3 citrate	3	2
pH at RT	5.8	5.8

RBC unit preparation

Units of blood (450 ± 45 mL) were collected in CP2D and held for 7 hours at room temperature (RT).

AS-24 units (WBC-reduced) held for 8 weeks. Packed RBCs were prepared by centrifugation (5000 × *g*, 5 min, RT) followed by the removal of plasma to achieve a target storage Hct of 65 percent. AS-24 (100 mL) was added, and this step was followed by RT filtration to remove WBCs by using an integral filter (Leukotrap RC system, Medsep Corp.) and gravity flow.

AS-3 units (WBC-reduced) held for 8 weeks. Whole blood was filtered at RT to remove WBCs by using an integral filter (Leukotrap) and gravity flow. Packed RBCs were prepared by centrifugation (5000 × *g*, 5 min, RT), and this step was followed by removal of the plasma. Finally, 100 mL of AS-3 was added.

AS-3 units (non-WBC-reduced) held for 6 weeks. Packed RBCs were prepared by centrifugation (5000 × *g*, 5 min, RT), and this step was followed by the removal of plasma to achieve a target storage Hct of 65 percent. Finally, 100 mL of AS-3 was added.

All units were gently mixed, sampled for in vitro testing, and placed in refrigerated storage (1-6°C) 8 hours or less after collection.

In vitro measurements

Samples from stored units were collected into a small pouch attached via a sterile connecting device (SCD 312, Terumo Medical Corp., Elkton, MD) to the remaining donor needle tubing. A battery of in vitro tests was performed on all units immediately before and after storage. Filtered units were also sampled before filtration.

Total Hb concentration, unfiltered WBC counts, and RBC counts were measured with a clinical hematology analyzer (Hematology Cell Counter System Series 9000, Baker, Allentown, PA). Supernatant Hb was measured spectrophotometrically (DU-62, Beckman, Fullerton, CA) using the modified Drabkin's assay.[13] The percentage of hemolysis was determined by the ratio of free Hb to total Hb. Centrifuged microhematocrits (Clay Adams, Becton Dickinson) were performed to avoid the readjustment of cell volume and subsequent errors caused by the isotonic diluent used in the blood analyzer. Mean corpuscular volume (MCV) was calculated from the centrifuged microhematocrit and the RBC count. Postfiltration WBC counts were taken by using a Neubauer hemocytometer and propidium iodide staining.[14] The RBC morphology score was determined according to the method of Usry et al.[15]

RBC ATP concentration was measured in deproteinized supernatants. Whole-blood or packed RBC aliquots were mixed with cold, 12-percent perchloric acid to precipitate blood proteins and centrifuged at 2700 × *g* for 10 minutes; then the protein-free supernatant was adjusted to pH 8 to 9 with solid $KHCO_3$ and frozen at –80°C until tested. ATP was assayed enzymatically by the use of a commercially available kit (Procedure 366-UV, Sigma Diagnostics, St. Louis, MO).

Check for bacterial contamination

Three or 4 days before the end of storage, sterilely collected aliquots from each unit were tested for bacterial contamination in broth and agar cultures by using a commercial blood culture system (BBL Septi-Check, Becton Dickinson Microbiology Systems, Cockeysville, MD). Absence of bacteria growth in the incubated cultures after 72 hours was required before a sample from the unit was prepared for infusion to the donor.

In vivo RBC recovery and survival measurement

After 6 or 8 weeks of storage, in vivo RBC recovery was measured 24 hours after autologous infusion by using a double-radiolabel procedure. In brief, a sample of the stored blood was labeled with 20 µCi of ^{51}Cr. Concurrently, a fresh blood sample was collected from the volunteer and labeled with 25 µCi of ^{99m}Tc. Carefully measured aliquots of the radiolabeled RBCs were mixed and rapidly infused. Blood samples were collected at timed intervals during the 30 minutes immediately after the infusion and again at 24 hours, 7 days, and 14 days after infusion. The radioactivity of the samples was measured with a gamma counter (CliniGamma Counter, Model 1272, LKB, Turku, Finland).

Gamma emissions from ^{99m}Tc-radiolabeled cells were measured in the samples collected during the 30 minutes after infusion and used to determine an independent RBC volume. The activity from ^{51}Cr-labeled cells was measured in all of the samples and used to calculate the fractional survival of the stored RBCs.

Statistical analysis

Comparisons of means within the individual crossover trials were made with the paired *t* test. For comparisons between the crossover trials, the unpaired *t* test was used. The correlations between the morphology scores and viability and the RBC ATP concentrations and viability were performed by the Pearson method. Probabilities less than 0.05 were considered significant.

RESULTS

Volunteers

Eleven volunteers completed the first study, and 13 completed the second study. Two individuals participated in and completed both studies. The remaining 8 volunteers withdrew for a variety of reasons; in four cases, the reason(s) was related to the study. Two donors withdrew after unsuccessful phlebotomy, 1 after a tubing weld failure contaminated a unit, and 1 after infiltration of an infusion site.

Prestorage equivalence of groups

Initial characteristics of the blood units are presented in Table 2. The units contained an average of 206 ± 12 mL of RBCs at collection. WBC-reduced units lost approximately 6 percent of their RBCs in the filter, and 14 mL of RBCs was removed from all units for prestorage testing. An additional 4 mL was removed from each WBC-reduced unit to allow postfiltration WBC counting. Plasma was removed from the units to produce packed RBCs with a storage Hct of 65 percent. The units began storage with RBC ATP concentrations averaging 5.1 ± 0.2 µmol per g of Hb and negligible hemolysis, which were equal in all groups.

Hypotonic swelling

As indicated by a 14-percent increase in MCV, from 95.7 ± 4.0 fL to 109.5 ± 6.7 fL (shown in Table 2), the AS-24 solution produced significant cell swelling. However, AS-3, which is isotonic with an effective osmolality of 270 mOsm, also induced an increase in the MCV of the other units, which averaged 8 fL. During the storage period, the AS-24 cells showed the greatest loss of volume, about 4 percent.

WBC filtration

In the AS-24 units, the WBC-reduction filter reduced the WBC count from 6082 per µL to 0.63 per µL, a 4 log reduction, to an average of 2.3×10^5 WBCs per unit. The average time needed to filter the AS-24 units was 40 minutes, because of the higher Hct of the RBC component and the size of the swollen cells. The average filtration time for anticoagulated whole blood in the AS-3 units was 9 minutes, and the filter reduced the WBC numbers below the detection limits of our assay (0.1/µL or 3.5×10^4/unit). Despite the different preparative stages at which the AS-24 and AS-3

TABLE 2. Similarity of stored unit characteristics at the beginning of storage*

	First study (n = 11)		Second study (n = 13)	
	8 weeks, filtered, in AS-24	6 weeks, unfiltered, in AS-3	8 weeks, filtered, in AS-3	6 weeks, unfiltered, in AS-3
Hct (%)	64.9 ± 4.0	62.5 ± 3.1	68.4 ± 5.0	67.8 ± 1.9
MCV (fL)	109.5 ± 6.7†	101.2 ± 4.6	103.2 ± 7.0	101.3 ± 3.2
ATP (µmol/g Hb)	5.1 ± 0.3	5.1 ± 0.2	5.0 ± 0.5	5.2 ± 0.7
Hemolysis (%)	0.026 ± 0.020	0.016 ± 0.022	0.014 ± 0.030	0.012 ± 0.021
Morphology index	93.4 ± 3.1	93.5 ± 3.6	89.2 ± 4.0	88.7 ± 3.5

* Data presented as mean ± SD.

† Only the MCV in these AS-24 units was significantly greater than that in its paired control, as a result of the hypotonic solution.

units were filtered, both groups lost only 6 percent of all RBCs (23 g of concentrated AS-24 product vs. 35 g of whole blood for the AS-3 units). Both groups achieved WBC-reduction standards established in previous validation of the filters.

Poststorage hemolysis, RBC ATP concentrations, and RBC morphology

Hemolysis averaged less than 1 percent under all conditions of storage in both studies (Table 3). WBC-reduced units in each study had significantly less hemolysis, despite 2-week longer storage. RBC ATP concentrations were lower in the units stored for 8 weeks in both studies than those in cells from the same donors that were stored for 6 weeks in AS-3. Despite the slight baseline differences in the two crossover studies and 2 additional weeks of storage for the 8-week units, there was no significant difference in the morphology scores of the four groups after storage.

Poststorage RBC viability

The 24-hour recovery fraction measures for the two studies are illustrated in Fig. 1. The mean 24-hour survivals for the 6-week AS-3 "control" arm of each study were equivalent at 77 percent and greater than the FDA standard of 75 percent. The 8-week arms of each trial had significantly lower survivals, at 64 ± 3 percent for the AS-24 units and 67 ± 2 percent for the AS-3 units. For all units, about 80 percent of the RBCs surviving 24 hours persisted in the circulation for 14 days.

The relationship of RBC morphology score and RBC ATP concentration to RBC viability

An analysis of the relationship between the RBC morphology score and viability showed a significant correlation, p = 0.001. However, the correlation explained only 21 percent of the relationship (Fig. 2). No morphology score defined a clear threshold for predicting adequate in vivo survival. RBC morphology scores of 60 or greater were counted for 9 of the 48 units; yet, for 4 of these, the 24-hour survival values were below 75 percent. Likewise, of 19 units for which viability studies met or exceeded 75-percent recovery, morphology scores for 12 were below 60. The stored blood unit with the highest 24-hour recovery fraction (95%) had a morphology score of only 55.

The correlation between RBC ATP concentration and RBC viability was stronger, accounting for 40 percent of the variability in the relationship (Fig. 3). A decrease of 1 µmol per g of Hb in the RBC ATP concentration at the end of storage is expected to reduce viability by 8.3 ± 1.5 percent, according to the observed regression coefficient. However, the positive predictive value of an RBC ATP concentration of 2 µmol per g of Hb or greater was weak. Only 19 of the 41 units with RBC ATP concentrations meeting this criterion exhibited viability of 75 percent or greater, but all 7 units below that threshold demonstrated unacceptably low viability. Only 12 of 17 units with an RBC ATP concentration equal to or greater than 3 µmol per g of Hb had a 24-hour survival of 75 percent.

TABLE 3. Differences in stored unit characteristics at the end of storage*

	First study (n = 11)		Second study (n = 13)	
	8 weeks, filtered, in AS-24	6 weeks, unfiltered, in AS-3	8 weeks, filtered, in AS-3	6 weeks, unfiltered, in AS-3
MCV (fL)	105.6 ± 4.8†	100.1 ± 5.3	100.9 ± 4.2	100.3 ± 3.7
ATP (µmol/g Hb)	2.0 ± 0.5†	3.1 ± 0.6	2.5 ± 0.5†	3.2 ± 0.6
Hemolysis (%)	0.31 ± 0.15†	0.49 ± 0.31	0.20 ± 0.07†	0.51 ± 0.39
Morphology index	53.7 ± 4.5	55.8 ± 5.3	52.6 ± 4.6†	59.9 ± 7.2

* Data presented as mean ± SD.
† Significantly different from their paired controls at the p<0.05 level. The lower hemolysis in the filtered units is probably clinically important, as it occurred despite the units being stored for 2 weeks longer and having lower RBC ATP concentrations and recovery.

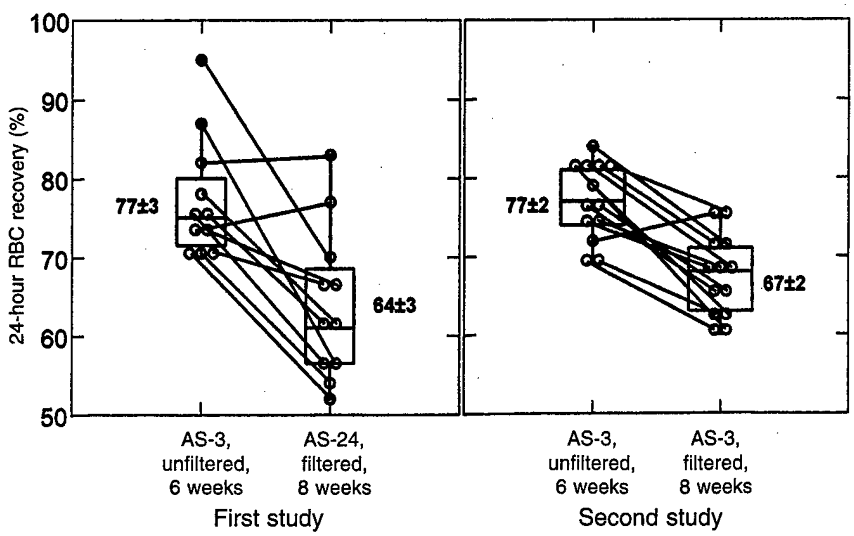

Fig. 1. The distribution of RBC recovery measures in the two studies. The paired measures are connected and the mean values are presented as mean ± SD.

DISCUSSION

In this investigation, we set out to test the prediction that a hypotonic AS (AS-24) would increase the viability of stored RBCs. Our paired crossover study design produced initially equivalent groups, and AS-24 did produce hypotonic swelling of the stored RBCs. Nevertheless, the viability was not usefully prolonged. Viability after 8 weeks' storage in AS-24

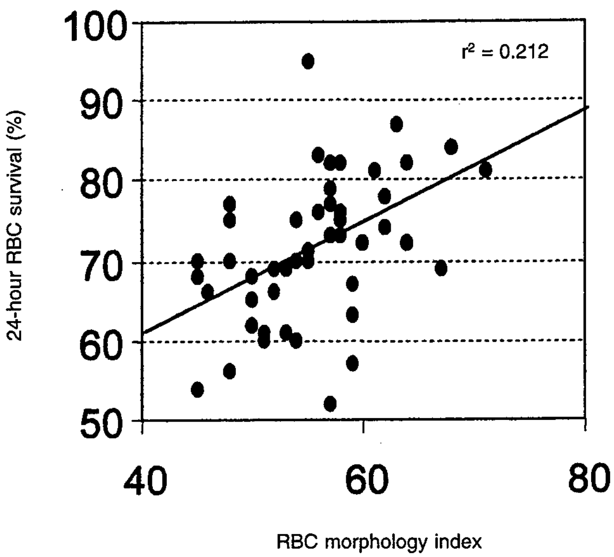

Fig. 2. The relationship of the RBC morphology index to the in vivo RBC recovery fraction. The prestorage morphology scores are very similar in all arms of the study and show no effect from storage in either solution to the recovery point.

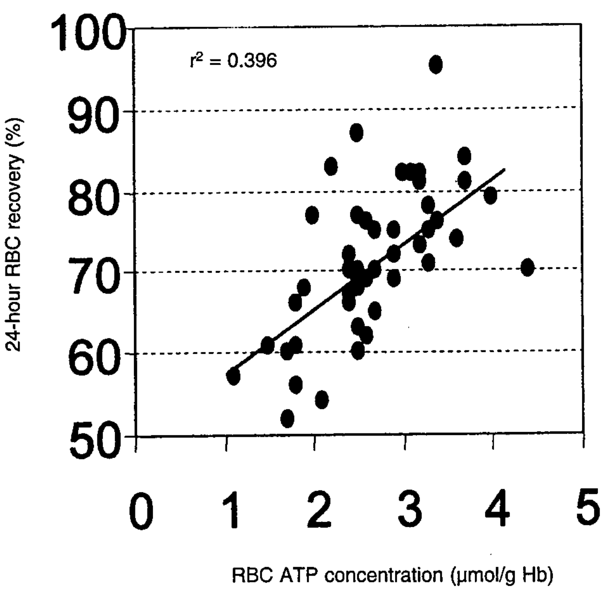

Fig. 3. The relationship of the RBC ATP concentration to the in vivo RBC recovery fraction. The RBC ATP concentration demonstrates better correlation with in vitro RBC recovery than with in vivo recovery (see Fig. 2).

appeared to be no better than that after 8 weeks' storage in AS-3 when the RBCs were stored under otherwise equivalent conditions. WBC-reduction by filtration decreased RBC hemolysis by one-half during storage in the 8-week arms of both trials, despite the additional 2 weeks of storage. There appeared to be no additional effect on reducing hemolysis conferred by storage in AS-24. Further, the RBC morphology scores of the WBC-reduced units stored for 8 weeks were equal in the trials and equivalent to those of the non-WBC-reduced units after 6 weeks of storage. In sum, the studies described here provide no evidence that the hypotonic AS (AS-24) improved viability, increased RBC morphology scores, or reduced hemolysis.

The 24-hour recovery of the RBCs stored for 6 weeks in AS-3 is lower than that in some previous reports,[10,16] but it is not significantly different from that reported by Arduini et al.[17] This lower-than-usual recovery appears to represent a particularly low subset within the range of viability measurements to be expected with a randomly chosen group of volunteers. The decrease in RBC ATP concentration with time between the endpoints of the 6- and 8-week arms was about 0.4 μmol per g of Hb per week, which is typical of the decrease seen in other storage studies with repeated sampling of RBC units.[18] The decrease in measured viability of 10 to 13 percent between 6 and 8 weeks is approximately what would be predicted from the decrease in RBC ATP concentration. The results suggest that AS-24 is no better than AS-3 when used in the 100-mL AS format.

In their original description of hypotonic ASs, Meryman et al.[2] showed that RBCs, stored at a Hct of 35 to 40 percent with an initial pH of 7.1 in a hypotonic solution containing high concentrations of ammonium, potassium, phosphate, and membrane protectants such as mannitol and citrate, contained viable cells for as long as 19 weeks. The report was important, because it showed that such extended storage was possible. The most successful of their solutions, "Solution 6," caused RBC ATP concentrations to rise to 160 percent of starting values at 4 weeks of storage. RBC ATP concentrations declined steadily thereafter, at about 10 percent of the starting value (0.4 μmol/g Hb) each week.

Four groups have subsequently reexamined this concept. In 1990, Greenwalt and colleagues[5] confirmed the increased RBC ATP concentrations with storage in Solution 6 in split-unit studies. Although they could also confirm better RBC morphology scores with storage in the hypotonic media than with storage in AS-1, they could not confirm RBC swelling by optical methods, and the loss of Hb in microvesicles was the same under both conditions of storage.[19,20] In another 1990 report, Mazor et al.[6] replaced the potassium and ammonium in Solution 6 with sodium and with rubidium, another monovalent cation, and found no difference in the initial rise in RBC ATP concentrations. In a 1994 report,[7] the same group found that hypotonicity did not affect the RBC ATP concentration, but that initial pH, phosphate concentration, and adenine concentration were important. This group performed no studies of RBC viability. In 1992, Kay and Beutler[8] also confirmed the ability of Solution 6 to increase RBC ATP concentrations. They concluded that ammonium was important because it disinhibited phosphofructokinase. However, they could not demonstrate improved viability with the storage of rabbit RBCs in Solution 6. Finally, in 1996, Dumaswala and colleagues[9] reported a comparison of two hypotonic media, one with phosphate and one without. The less hypotonic medium with and a higher initial pH had better preservation of RBC ATP concentrations.

The demonstration of 14-week RBC storage by Meryman et al. has always been clouded by the recognition that, "[f]rom the clinical standpoint, Solution number 6 would clearly be unacceptable for transfusion…."[2(p505)] Attempts to understand and translate this work into clinically useful storage solutions have been limited by the failure to measure clinically relevant endpoints, such as human RBC viability and storage hemolysis, with reasonable statistical power. Future work in this field should attempt to maximize RBC ATP concentrations by the manipulation of pH, adenine, and phosphate content and to minimize hemolysis by WBC-reduction. Progress can be made with candidate ASs and storage systems incorporating these innovations.

ACKNOWLEDGMENT

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Appendix C - (Successful storage of RBCs for nine weeks in a new additive solution)

Successful storage of RBCs for 9 weeks in a new additive solution

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BACKGROUND: This study explored the effect of storing packed RBCs suspended in 200 mL of an alkaline, hypotonic, experimental additive solution (EAS 61).
STUDY DESIGN AND METHODS: Packed RBC units prepared from RBCs collected from healthy donors in CPD were stored for 8 (n = 10) and 9 (n = 10) weeks under blood bank conditions after the addition of 200 mL of EAS 61 (adenine, 2 m*M*; dextrose, 110 m*M*; mannitol, 55 m*M*; NaCl, 26 m*M*; Na_2HPO_4, 12 m*M*). Standard methods were used for in vitro assays. The 24-hour in vivo autologous recoveries were measured with ^{51}Cr.
RESULTS: Mean ± SD recoveries at 8 and 9 weeks were 81 ± 7 and 77 ± 7 percent. After 9 weeks, the ATP of the RBCs was 81 percent of the initial value, hemolysis was 0.35 percent, supernatant potassium was 46 mEq per L, and the morphologic index was 94.1.
CONCLUSION: Packed RBCs suspended in 200 mL of EAS 61 can be stored satisfactorily for 9 weeks. Longer RBC storage should reduce outdating, increase availability of transfusions in remote locations, and improve the efficiency of autologous donor programs.

ABBREVIATIONS: EAS = experimental additive solution; MCV(s) = mean corpuscular volume(s).

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We have reported 73 percent recovery of RBCs stored for 9 weeks in 200 mL of a hypotonic experimental additive solution (EAS) containing glycerol (150 m*M*) and disodium phosphate.[1] Only 61 percent of the RBCs in the other half of the same units were recovered when stored for the same time in 100 mL of another additive solution (Adsol, Baxter Healthcare, Roundlake, IL). Review of these split-unit studies suggested that the glycerol, originally added to reduce hemolysis, may have impaired metabolism. Disodium phosphate may have been the more important ingredient, because it both provided inorganic phosphate and raised the storage pH. The additional volume also appeared to be important. The purpose of this report is to record the results of studies of packed RBCs stored for 8 and 9 weeks in 200 mL of a similar EAS containing saline, adenine, dextrose, mannitol, and inorganic phosphate, but no glycerol.

MATERIALS AND METHODS

Study design, volunteers, and storage solution

This was an uncontrolled trial of prolonged RBC storage. Twenty units were stored in all: 10 units for 8 weeks and 10 for 9 weeks. They were stored upright in holders under standard blood bank conditions. The major measured outcome was the 24-hour autologous in vivo RBC recovery fraction, the proportion of the stored cells still in the circulation 24 hours after infusion of the RBCs to the original donor. This was measured by the single-isotope technique. This technique involves the estimation of the RBC mass and the 24-hour recovery fraction from the dilution of the ^{51}Cr-labeled stored cells. Several in vitro measures were performed on the stored RBCs as well.

Volunteers acceptable as blood donors by AABB and FDA criteria were used. The protocols were approved by the University of Cincinnati's Institutional Review Board.

A pyrogen-free, sterile EAS (EAS 61) was prepared as described previously.[2] Briefly, the recipe (adenine, 2 m*M*; dextrose, 110 m*M*; mannitol, 55 m*M*; NaCl, 26 m*M*; Na_2HPO_4, 12 m*M*; pH 8.23 at 25°C and total osmolality, 254 mOsm/kg) were mixed in sterile water for injection in a laminar flow hood and sterilely filtered into 1000-mL blood transfer bags. The bags were sampled by use of a sterile connecting device (SCD 312, Terumo, Elkton, MD), and the samples were tested with bacterial culture and the Limulus assay for bacterial endotoxins. The solution bags were held for 2 weeks to ensure that bacterial cultures were without growth and then aliquoted by weight by sterile transfer into sterile blood storage bags.

Handling of blood units

Standard units of blood (450 ± 45 mL) were collected with 63 mL of CPD solution in a triple-bag collection system (4R1402, Baxter Healthcare Corp., Deerfield, IL). Packed RBCs were prepared after centrifugation for 10 minutes as described previously.[2] Two hundred mL of EAS 61 was added to the units of packed cells by use of the sterile connecting device.

Units were undisturbed except for sample removals at 1 and 2 weeks for 2,3-DPG determination and 1 week before the recovery studies for bacterial culture.[2] All samples were obtained by using the sterile connecting device.

Measurement of 24-hour in vivo autologous RBC recovery

On the last day of storage, a 10-mL sample was labeled with approximately 15 µCi of ^{51}Cr, as recommended by the International Committee on Standardization in Hematology[3] and Moroff et al.,[4] and infused into the original donor. Blood samples were drawn at 5, 7.5, 10, 12.5, 15, 20, and 60 minutes and 24 hours after infusion for gamma counting for the ^{51}Cr recovery study. The ^{51}Cr-labeled RBCs were counted on an automated gamma counter (Cobra II, Packard Instrument Co., Meriden, CT).

Other studies performed on RBCs stored in EAS 61

The methods used for the hematologic studies—measurement of ATP, 2,3-DPG, potassium, pH, and percentage hemolysis—have been described previously.[2] The mean corpuscular volumes (MCVs) and mean corpuscular Hb concentrations (MCHCs) were calculated by using spun Hcts and electronic hemograms (MaxM, Coulter Electronics, Hialeah, FL). The RBC morphology index was measured by a modification of the method of Usry et al.[6]

Statistics

Means and SDs were calculated as descriptive statistics within the 8- and 9-week storage groups. Box plots were used to display the data.[7]

RESULTS

All 20 volunteers successfully completed the study. The volunteer cohort is unusual, in that 16 of the 20 volunteers were female and many were overweight with a mean ponderal index (weight over height squared) of 28.5.

The in vitro data for the RBCs stored in EAS 61 for 8 (n = 10) and 9 (n = 10) weeks are shown in Table 1. Specifically in regard to the 9-week storage data, of note are the high poststorage RBC ATP concentrations, which averaged 3.5 ± 0.6 µmol per g of Hb (mean ± SD). The initially very high MCVs, which decreased during storage, were the result of osmotic swelling of the RBCs from the hypotonic additive solution and the volume decrease that followed. RBC morphology was well preserved, and hemolysis was low at 0.35 ± 0.18 percent after 9 weeks of storage.

The in vivo recovery studies are described in detail in Table 2 and summarized in Fig. 1. The 24-hour single-label autologous RBC recoveries at 8 weeks ranged from 66 to 87 percent, with a mean of 81 ± 7 percent, and, at 9 weeks, they ranged from 61 to 83 percent, with a mean value of 77 ± 7 percent.

DISCUSSION

We have developed an additive solution that allows the storage of human RBCs for 9 weeks with a measured in vivo recovery of greater than 75 percent and hemolysis of less than 0.5 percent. These storage times are greater than those obtainable with any of the currently licensed storage solutions. Moreover, the RBCs maintain high concentrations of ATP and excellent morphology during storage.

Recovery measurements are by nature imprecise.[8] They contain Poisson distribution errors in isotope counting and significant random errors in the measurement of the microhematocrit. They can also contain systematic er-

TABLE 1. In vitro and in vivo data on RBC units after storage*

	8-week storage (n = 10)			9-week storage (n = 10)		
	Before storage	1 hour	8 weeks	Before storage	1 hour	9 weeks
ATP (µmol/g Hb)†	4.00 ± 0.23	3.97 ± 0.28	3.29 ± 0.32	4.28 ± 0.43	4.20 ± 0.34	3.47 ± 0.58
Hemolysis (%)	0.03 ± 0.01	0.03 ± 0.01	0.26 ± 0.10	0.04 ± 0.02	0.03 ± 0.00	0.35 ± 0.18
Extracellular pH	7.30 ± 0.04	7.37 ± 0.08	6.55 ± 0.08	7.31 ± 0.03	7.42 ± 0.11	6.48 ± 0.05
Intracellular pH	7.21 ± 0.02	7.20 ± 0.02	6.49 ± 0.05	7.18 ± 0.03	7.19 ± 0.04	6.47 ± 0.04
Supernatant Potassium (mEq/L)	4.7 ± 0.3	2.2 ± 0.2	44.9 ± 3.0	4.70 ± 0.47	2.31 ± 0.53	46.0 ± 2.9
Spun Hct (%)	38 ± 2	54 ± 1	48 ± 1	38 ± 4	54 ± 2	48 ± 2
MCV (fL)	95.9 ± 8.0	121.8 ± 12.4	107.0 ± 9.9	97.0 ± 2.9	123.7 ± 15.4	106.6 ± 4.1
Morphologic index	99.4 ± 0.5	98.4 ± 0.8	94.6 ± 6.7	99.0 ± 1.2	99.2 ± 1.1	94.1 ± 2.9

* 2,3-DPG levels (µmol/g Hb; mean ± SD: 8-week storage: before storage, 14.32 ± 1.33; at 1 week, 9.13 ± 1.53; at 2 weeks, 1.63 ± 0.90; 9-week storage: before storage, 13.82 ± 2.68; at 1 week, 8.07 ± 1.92; at 2 weeks, 1.62 ± 1.13.

† Percentage of initial level: for 8-week storage, 82.25 percent; for 9-week storage, 81.07 percent.

TABLE 2. Characteristics of the volunteers and their 24-hour autologous RBC recovery studies

Volunteer number	Age/Sex	Ht/Wt (m/kg)	Hb/Hct in CPD	Blood volume[9] (mL)	RBC volume ^{51}Cr (mL)	^{51}Cr recovery (%)
8-week study						
1	31/F	1.70/104	10.8/37	5374	1890	74
2	48/F	1.57/91	11.1/36	4572	1727	87
3	47/F	1.63/69	12.6/39	4008	1236	66
4	41/F	1.78/76	12.1/39	4706	1624	82
5	40/F	1.63/63	12.6/39	3810	1464	84
6	59/F	1.65/100	12.9/36	5092	1720	86
7	21/M	1.78/93	12.8/40	5667	2237	85
8	28/F	1.68/82	12.3/40	4585	1677	79
9	51/F	1.73/113	11.1/35	5765	2051	80
10	44/M	1.85/132	13.0/41	7176	2748	85
Mean ± SD				5075 ± 983	1837 ± 426	81 ± 7
9-week study						
11	51/F	1.63/73	12.2/39	4141	1525	79
12	56/F	1.73/70	10.5/35	4343	1478	81
13	40/F	1.68/66	11.3/36	4055	1359	73
14	55/F	1.73/67	12.3/39	4244	1626	75
15	32/F	1.63/50	11.5/37	3380	1367	83
16	30/F	1.70/68	10.7/35	4183	1204	61
17	27/M	1.83/77	13.8/42	5331	2144	83
18	55/M	1.90/95	14.3/47	6179	2661	78
19	28/F	1.80/125	12.3/40	6396	2029	79
20	46/F	1.60/82	10.3/34	4355	1351	81
Mean ± SD				4661 ± 980	1674 ± 460	77 ± 7

rors, such as the early loss of senescent RBCs before entry into the circulation has taken place. An attempt to calculate an RBC volume on the basis of height and weight would have involved large errors.[9] We used the ^{51}Cr single-label measure of RBC volume to calculate the RBC recovery. Measures of RBC recovery by this single-label ^{51}Cr technique are said to be very accurate when the recovery is high,[8] as in this study. This RBC storage solution exceeds the current FDA standard for acceptability of greater than 75 percent recovery and less than 1 percent hemolysis.[8]

Our solution is composed of water, dextrose, adenine, mannitol, and disodium phosphate. It is used as a 200-mL volume added to RBCs prepared from a conventional unit of blood collected in 63 mL of CPD. The FDA-licensed additive solutions, AS-1 (Adsol), AS-3 (Nutricel, Medsep, Covina, CA), and AS-5 (Optisol, Terumo Medical, Somerset, NJ), are all approved for 6 weeks' storage of RBCs, and each has been shown to have less than 75 percent recovery at 7 weeks.[10-14] EAS 61 differs from these licensed additive solutions in two ways. First, the disodium phosphate is unbuffered, and, second, the volume of the storage solution is increased from 100 to 200 mL.

The addition of disodium phosphate results in a solution with a pH of 8.3; after mixing with the packed RBCs at room temperature, the pH is about 7.3. The increased concentration of adenine resulting from the greater volume of additive solution used and the presence of the increased concentration of inorganic phosphate supports an increased synthesis of ATP during the first 4 weeks of storage. This was shown in two in vitro experiments using EAS 61.[15] The increased ATP synthesis led to RBC ATP concentrations of 110 percent and 113 percent of the initial value after 2 weeks' storage; after 4 weeks' storage, they were 104 percent and 103 percent. In these in vitro studies, the units were mixed at weekly intervals to permit sampling. In the 8- and 9-week in vivo studies reported here, the 20 units were undisturbed, except for the removal of a sample for culture a week before infusion, and the RBCs were in high concentration at the bottom of the bag for most of the time of storage. Nevertheless, the RBC ATP concentration after 9 weeks' storage was 81 percent of the initial value.

Increasing the volume of the storage solution from 100 mL to 200 mL produced a marked effect. In one of the in

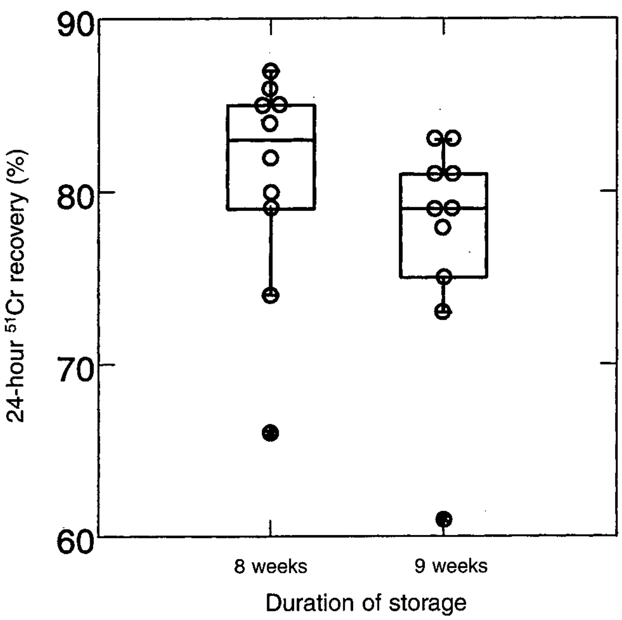

Fig. 1. Box-and-whisker plots of the 24-hour recoveries. Distribution of 24-hour single-isotope recoveries in the 8- and 9-week studies. The box marks the 25- to 75-percentile data points and is a measure of the spread of the middle half of the data. Box inner line marks the median. Whiskers extend to the highest and lowest values that fall within 1.5 times the box height from the box edge or 1.0 time the box height from the next closest value. Asterisks mark values outside these limits. In RBC viability studies of this kind, 1 in 10 to 1 in 40 volunteers will typically have a low outlying value, an RBC recovery substantially lower than usual. We have observed that, in some of these individuals whose RBCs store poorly, RBC ATP concentrations are not maintained under storage conditions.

vitro studies mentioned above, aliquots of pools of RBCs of the same ABO and Rh type were stored in 200 mL and 100 mL of EAS 61. In these samples, the respective RBC ATP concentrations were 103 percent and 88 percent of the initial concentration after 4 weeks of storage. As would be expected with the higher ATP concentrations, the morphologic indices were better maintained when the Hct during storage was 45 percent rather than 55 percent. The morphologic index after 9 weeks was 80.3 for the RBCs stored with 100 mL of additive and 92.0 for those stored with 200 mL. In the present study, the morphologic index was 94.1 after 9 weeks of storage, which agrees well with the previous in vitro observations and indicates that most of the RBCs retained their discoid shape during storage.

This additive solution should not create any practical clinical or manufacturing problems. The extra 100 mL of additive solution results in a lower storage Hct of about 0.45, compared to 0.55 to 0.60 with the licensed additive solutions. The additional volume will not make a clinically significant difference, even in massive transfusions. A patient receiving 10 units of packed RBCs would get an extra liter of water and 24 more g of mannitol than with AS-1 (Adsol), but no more phosphate than with AS-3 (Nutricel). The mixture cannot be heat-sterilized in an oxidative environment without caramelization of the dextrose, but this problem has been circumvented by compartmentalizing the components in the manufacture of other neutral or alkaline additive solutions such as ErythroSol (Baxter International, Orth, Austria)[16] and AS-17 (an EAS prepared by Miles-Cutter).[17]

There are four obvious benefits and one potential benefit from prolonged storage of RBCs. The first is that, currently, more than 5 percent of RBC units nationwide are outdated.[18,19] Reduced losses resulting from longer liquid storage would help to mitigate national seasonal shortages. Second, longer storage would allow more generous emergency stocks of blood in remote locations. This is especially important to the military, which must maintain blood depots for contingency use at several places around the world. It is also important for remote communities, such as in American Samoa, where one of the authors (JRH) served as director of health. Third, the utility of autologous donations is limited by the relatively short storage life of liquid-stored RBCs. Nine-week storage would make the process more efficient. Fourth, for the typical, short storage times of RBCs in modern blood centers, better storage should translate to a reduced burden of unrecoverable cells with each transfusion. Fifth, it has been shown that the breakdown products released from RBCs during storage, such as the phospholipid platelet-activating factor, contribute to the pathogenesis of transfusion reactions such as transfusion-related acute lung injury and multiple organ failure.[20] These better preserved RBCs may release less platelet-activating factor.

We have developed a 9-week additive solution (EAS 61) that is compatible with present manufacturing and clinical practice. It has the potential to save the approximately 300,000 units of RBCs that otherwise would outdate annually, to improve the logistics of supplying blood to remote areas, to make autologous transfusion programs more efficient, and to improve the safety and effectiveness of blood transfusions.

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Appendix D - (Successful storage of RBCs for ten weeks in a new additive solution)

Successful storage of RBCs for 10 weeks in a new additive solution

J.R. Hess, N. Rugg, A.D. Knapp, J.F. Gormas, E.B. Silberstein, and T.J. Greenwalt

BACKGROUND: The effect of storing packed RBCs suspended in 300 mL of an alkaline, experimental additive solution (EAS 64) was explored.

STUDY DESIGN AND METHODS: RBC units prepared from blood collected from healthy donors into CPD were WBC reduced and stored for 10 weeks under blood bank conditions after the addition of 300 mL of EAS 64 (adenine, 2 m*M*; dextrose, 50 m*M*; mannitol, 20 m*M*; NaCl, 75 m*M*; Na_2HPO_4, 9 m*M*). For comparison, non-WBC-reduced units from the same donors were stored in a different additive solution (AS-1, Baxter Healthcare) for 6 weeks. Standard methods were used for the in vitro assays. The 24-hour in vivo recoveries were measured by using ^{51}Cr- and ^{99m}Tc-labeled RBCs.

RESULTS: Mean recovery in the EAS 64 units after 10 weeks was 84 ± 8 percent, the same as in the AS-1 units stored for 6 weeks. For EAS 64 and AS-1 units, respectively, the ATP of the RBCs was 85 percent and 64 percent of the initial value, hemolysis was 0.43 percent and 0.63 percent, supernatant potassium was 24 mEq per L and 44 mEq per L, and the morphologic index was 98 and 71.

CONCLUSION: RBCs suspended in 300 mL of EAS 64 can be stored satisfactorily for 10 weeks. Longer RBC storage should reduce outdating, increase availability of transfusions in remote locations, and improve the efficiency of autologous donor programs.

Longer RBC storage will benefit both blood bankers and patients.[1] Blood bankers will benefit from greater inventory, reduced outdating, and less frequent need for shipping to maintain stocks in remote locations. Patients will benefit by receiving fewer nonviable cells and RBC breakdown products in each unit, as well as by having the opportunity to participate in markedly improved autologous blood storage systems.

Improving blood storage solutions offers the most obvious way to lengthen RBC liquid storage. The standard volumes for current blood storage, 63 mL for anticoagulant and 100 mL for additive solutions, came from military research and the need to keep the weight of the blood component low for air shipment.[2] These standards persist, despite the demonstration that increased storage Hct is associated with reduced RBC viability.[3] Even the military requirement for low-weight blood components has been reduced by the use of better aircraft and can be traded for longer shelf-life.

We previously reported that RBCs can be stored for 9 weeks in an alkaline, hypotonic, phosphate-containing, experimental additive solution (EAS), EAS 61, when the additive solution was used in a 200-mL volume[4] (see also accompanying article, beginning on page 1007 of this issue

ABBREVIATIONS: EAS = experimental additive solution; MCV(s) = mean corpuscular volume(s).

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of TRANSFUSION). Further, we had shown that the quality of the stored RBCs, measured by increased ATP concentrations, better morphology scores, and reduced hemolysis, was even better maintained in greater volumes of the additive solution.[5] To take advantage of this effect of additive solution volume for longer storage, we reformulated EAS 61 as EAS 64 for 300-mL use, reducing the concentrations of dextrose, mannitol, and phosphate. In this report, we show that the EAS 64 solution yields excellent RBC viability and minimal hemolysis for at least 10 weeks. The viability was demonstrated in a crossover study comparing 24-hour RBC recovery measured by the double-isotope method of RBCs stored for 6 weeks in AS-1 (Adsol, Baxter Healthcare, Roundlake, IL) with those stored for 10 weeks in EAS 64.

MATERIALS AND METHODS

Volunteers, study design, and RBC unit preparation

Whole units of blood from 24 donors acceptable by AABB and FDA criteria were used in an in vitro pooling study. The protocol was approved by the University of Cincinnati's Institutional Review Board, and the method has been described previously.[4] Briefly, packed RBCs from 4 units of identical ABO type were sterilely pooled in a 1000-mL bag, thoroughly mixed, and aliquoted as 4 pooled test units to reduce interdonor variability. To 1 unit from each of the six pools was added 100, 200, 300, or 400 mL of EAS 64. The pyrogen-free, sterile EAS was prepared as described previously.[6] Units were sampled sterilely 1 hour after preparation and weekly thereafter for 11 weeks. All samples were obtained by using a sterile docking device (SCD 312, Terumo, Elkton, MD). Units in this in vitro study were not WBC reduced.

Ten additional blood donors acceptable by AABB and FDA criteria were used in a nonrandomized, crossover, in vivo study. The protocol was approved by the University of Cincinnati's Institutional Review Board. Units of blood (450 ± 45 mL) were collected with 63 mL of CPD solution in plastic triple bags with an integral WBC-reduction filter (4R3303, Baxter Healthcare Corp., Deerfield, IL) and were WBC reduced shortly after collection. All the units were stored upright in holders under standard blood bank conditions. They were undisturbed except for sample removals after 1 hour at room temperature and 1 week before the recovery studies for bacterial and fungal culture, respectively.[7] After a waiting period of ≥8 weeks, 10 additional units were collected from the same donors in triple-bag assemblies without a WBC-reduction filter (4R1436, Baxter) and stored for 6 weeks after the addition of 100 mL of AS-1, but otherwise processed in a like manner.

Hematologic and chemical measurements

The methods used for the hematologic studies—measurement of ATP, 2,3-DPG, potassium, pH, and percentage of hemolysis—have been described previously.[7] The mean corpuscular volumes (MCVs) and mean corpuscular Hb concentrations were calculated by using spun Hcts and electronic hemograms (T660, Coulter Electronics, Hialeah, FL). RBC morphology index measures were performed by a modification of the method of Usry et al.[8] The quantity of total protein shed in microvesicles was determined as described previously.[9]

Measurement of 24-hour RBC recovery fraction

On the last day of storage, a 15-mL sample of the stored RBCs was labeled with approximately 15 µCi of ^{51}Cr, as recommended by the International Committee on Standardization in Hematology[10] and Moroff et al.,[11] and transfused to the original donor. Blood samples were drawn from the other arm at 5, 7.5, 10, 12.5, 15, 20, and 60 minutes and 24 hours after transfusion, for the ^{51}Cr recovery study. Before the return of the ^{51}Cr-labeled RBCs, a 3-mL heparinized blood sample was collected from the donor. Between the collection of the 20- and 60-minute samples for the recovery study, the 3-mL fresh blood sample was labeled with approximately 2 mCi of ^{99m}Tc (UltraTag Kit, Mallinckrodt Medical, St. Louis, MO).[12] This blood was injected immediately after the 60-minute ^{51}Cr sample was obtained. A sample to count the ^{99m}Tc to determine the whole-body RBC mass was drawn after 10 minutes. The ^{51}Cr and ^{99m}Tc were counted on an automated gamma counter (Cobra II, Packard Instrument Co., Meriden, CT).

Statistical methods

In the in vitro study, ANOVA was used to evaluate differences in RBC measures in relation to additive solution volume at the weekly time points. In the in vivo study, a *t* test was used. Any differences noted in means, while unlikely at $p<0.05$, were considered significant. Data were maintained and graphs produced with software (Excel, Microsoft Corp., Redmond, WA). The box-and-whisker plot was produced with software (Systat Version 6.1, SPSS, Chicago, IL).

RESULTS

Data from the in vitro pooling study are displayed in Fig. 1. Increasing the EAS 64 volume resulted in better preservation of RBC ATP concentration (Fig. 1A) and morphologic index (Fig. 1C), as well as reduced RBC hemolysis (Fig. 1D). The improvements in RBC ATP concentration observed by increasing the EAS 64 volume from 200 to 300 mL are approximately equivalent to losses observed by prolonging storage from 9 to 10 weeks.

The data from the in vivo study are summarized in Table 1 and Fig. 2. It is significant that the EAS 64 units' RBC ATP concentration was maintained at 85 ± 14% (CV, 16.5) at 10 weeks, while, in AS-1 units, RBC ATP concentrations were only 64 ± 8 percent (CV, 12.5) of the initial value at 6 weeks.

TABLE 1. In vitro and in vivo data on RBCs after storage*						
	EAS 64 (300 mL) for 10 weeks (WBC-reduced)			AS-1 (100 mL) for 6 weeks (non-WBC-reduced)		
	Before storage	1 hour	10 weeks	Before storage	1 hour	6 weeks
ATP (µmol/g Hb)	4.19 ± 0.36	4.70 ± 0.41	3.58 ± 0.66	4.45 ± 0.32	4.39 ± 0.39	2.86 ± 0.45
ATP % of initial	–	112 ± 4	85 ± 14	–	99 ± 4	64 ± 8
Hemolysis (%)	0.06 ± 0.02	0.06 ± 0.03	0.43 ± 0.23	0.07 ± 0.06	0.05 ± 0.02	0.63 ± 0.39
DPG (µmol/g Hb)	12.76 ± 2.03	12.72 ± 2.03	–	11.20 ± 1.77	10.93 ± 1.32	–
Extracellular pH	7.28 ± 0.04	7.26 ± 0.04	6.75 ± 0.04	7.31 ± 0.03	7.32 ± 0.04	6.75 ± 0.07
Potassium corrected (mEq/L)	4.5 ± 0.3	<1	24.1 ± 2.7	4.1 ± 0.3	1.7 ± 0.1	43.9 ± 4.1
Lactic acid (mmol/L)	3.8 ± 0.7	1.5 ± 0.3	16.4 ± 3.2	4.0 ± 0.7	3.5 ± 0.5	28.1 ± 2.0
Glucose (mg/dL)	370 ± 19	740 ± 31	574 ± 27	353 ± 10	948 ± 36	700 ± 33
Phosphate (mg/dL)	10.4 ± 0.6	28.1 ± 1.3	26.2 ± 1.4	10.5 ± 0.5	4.0 ± 0.5	16.4 ± 0.7
Spun Hct (%)	39 ± 2	41 ± 2	40 ± 1	38 ± 3	55 ± 2	56 ± 2
MCV (fL)	96 ± 6	117 ± 7	114 ± 6	97 ± 6	95 ± 4	97 ± 4
Morphologic index	99.0 ± 0.6	98.8 ± 0.2	98.4 ± 1.2	99.0 ± 1.1	94.4 ± 4.1	70.8 ± 6.8
Vesicle protein (mg/dL) RBCs	–	–	6.77 ± 2.8	–	–	31.53 ± 6.8
$^{51}Cr/^{99m}Tc$ recovery (%)	–	–	84 ± 8	–	–	84 ± 8

* n = 10; values given as mean ± SD.

Hemolysis was less than 0.5 percent, and the morphologic index indicates that RBC crenation was minimal in EAS 64 units after 10 weeks of storage. The total microvesicle protein shed by RBCs in EAS 64 units during 10 weeks was 6.77 ± 2.8 mg per dL of RBCs, whereas the value after storage for 6 weeks in AS-1 units was 31.53 ± 6.3. The 24-hour in vivo recoveries were essentially identical at 84 ± 8 percent for both the EAS 64 units after 10 weeks of storage and the AS-1 units stored for 6 weeks (CV for both sets of values, 9.5).

DISCUSSION

We have developed an EAS that allows the storage of human RBCs for 10 weeks with in vivo 24-hour RBC recovery fractions of greater than 80 percent and with hemolysis of less than 0.5 percent. This EAS 64 is composed of saline, adenine, glucose, mannitol, and disodium phosphate in 300 mL of deionized water. The solution is directly infusable, the unit content of the individual compounds is compatible with massive transfusion, and all the compounds are used in similar or higher concentrations in additive solutions licensed in North America or Europe.

This EAS apparently works to improve RBC storage by two separate mechanisms. First, the higher pH and adenine and phosphate concentrations initially drive RBC ATP synthesis.[13] Increases in RBC ATP concentration are measurable by 1 hour after mixing and persist for the duration of useful storage. RBC ATP concentrations are an important determinant of RBC viability.[14] Second, there is a direct effect of storage solution volume on reducing RBC hemolysis. This effect is independent of RBC ATP concentration and osmolality, for it occurs with AS-1, which is mildly hypertonic and which causes lower RBC ATP concentrations with increasing AS-1 volume.[15]

WBC reduction also helps to increase RBC viability and reduce hemolysis. Heaton and colleagues[16] demonstrated that WBC reduction can increase the RBC 24-hour recovery after 6 weeks of storage by about 4 percent. Greenwalt and colleagues[9] and Babcock and colleagues[14] showed that WBC reduction can reduce hemolysis by at least 50 percent after 6 to 8 weeks of storage. Presumably, WBC reduction improves viability by removing WBCs with high metabolic activity, leading to better preservation of pH and adenine concentrations. It probably reduces hemolysis by removing WBCs before they break down to release enzymes that attack the RBC membrane.[17] WBC reduction was used in conjunction with EAS 64, because this appears likely to become a standard practice and because it seemed likely to work synergistically with the EAS to improve RBC storage.

Additional studies of this novel storage system will be required to confirm and extend the results presented here. One study reproducing the in vitro results of storage in 300 mL of EAS 64 performed in a second laboratory is reported elsewhere in this issue of **TRANSFUSION**.[5] Further work will be necessary to confirm the stability of RBC antigens during 10 weeks of storage. Studies in other laboratories will be needed to confirm that the results observed are not the result of an unusual volunteer cohort or other uncontrolled effects, such as unnoticed differences in storage temperature. Finally, if the solutions are to be used in routine blood banking, they will have to be commercially manufactured, tested, and licensed for use. Nevertheless, the results are promising.

The use of EAS 64 is compatible with contemporary blood bank methods. RBCs collected into 63 mL of CPD were WBC reduced, and 300 mL of EAS 64 was added. The increased volume resulted in an in-the-bag Hct of 0.40 ± 2. This value is 0.04 higher than that predicted on the basis of simple volume dilution of 200 mL of RBCs into 350 mL of additive solution and plasma, because of the RBC swelling associated with the hypotonic EAS. EAS 64 has half the sodium chloride concentration of AS-1. The use of disodium phosphate rather than the monosodium salt results in a pH of 8.4 in the EAS. The pH of the stored blood is 7.28 in the RBC supernatant immediately after mixing when measured at room temperature.

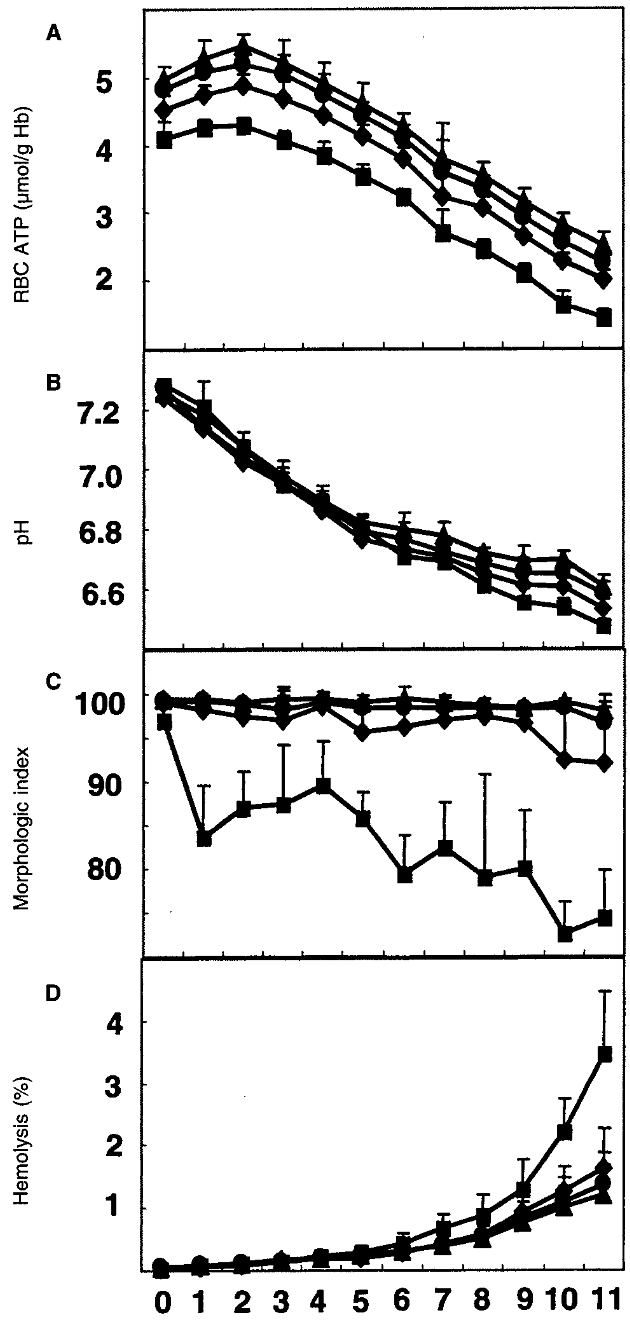

Fig. 1. A) RBC ATP concentrations, B) supernatant pH, C) morphologic index scores, and D) fractional hemolysis of six pools of RBCs stored in 100 (—■—), 200 (—◆—), 300 (—●—), and 400 (—▲—) mL of EAS 61. For each measure, there is a significant improvement with increasing volume of additive solution. EAS 64 was designed to take advantage of these relationships for 10-week RBC storage while delivering a minimum of total content of dextrose, mannitol, and phosphate.

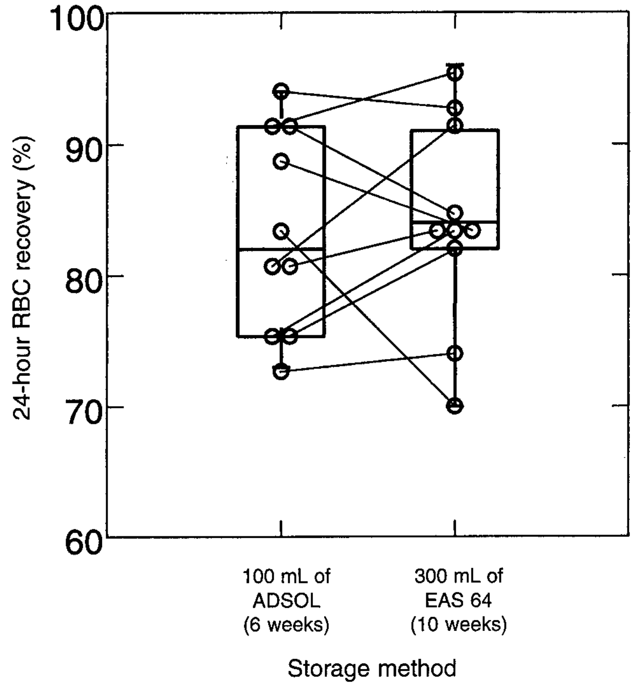

Fig. 2. Box-and-whisker plot of 24-hour RBC recovery fractions. Distribution of the 24-hour $^{99m}Tc/^{51}Cr$ double-label RBC recovery fractions. The two values for each donor are connected. The box marks the 25- to 75-percentile data points. The box inner line marks the median. Whiskers mark the highest and lowest values that lie within 1.5 times the interquartile distance (the box height) from the next closest value.

This EAS should not create any practical clinical problems. The osmolality of the stored blood solution is about 270 mOsm, very like that of normal humans and unlikely to cause clinical problems in pediatric or massive transfusion situations. The functional storage Hct of 0.36 is well above modern transfusion triggers, and the additional amounts of water, salt, and dextrose are small compared to those given in resuscitation situations.[18,19] The total mannitol and phosphate concentrations are one-half to one-third those of other licensed solutions, so the total content is only slightly greater.[20] The adenine content, once considered worrisome, is now known to be safe under almost all conditions.

The solution should not create any significant manufacturing problems. While alkaline dextrose solutions cannot be heat-sterilized because the sugar caramelizes, this problem has been overcome in other licensed and experimental additive solutions such as ErythroSol (Baxter International, Orth, Austria) in Europe and AS-17 (an EAS prepared by Miles-Cutter) by separating the glucose and phosphate during autoclaving.[21,22] In a multiple-bag system designed for collecting WBC-reduced RBCs, platelets, and plasma, such separation is easily achieved.

The advantages of 10-week RBC storage are multiple and include reduced outdating, reduced seasonal shortages, improved supply of remote locations, and more effective autologous blood-donation programs.[1] The safety advantages of storage systems that cause less hemolysis and membrane loss are not proven at this time, but are likely and deserve evaluation.[23] The interplay between the potential lengthening of RBC storage time and improvement of RBC product safety that might be possible with storage solutions such as EAS 64 creates exciting possibilities and calls for much additional work.

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Appendix E - (Eleven week blood storage)

RBC storage for 11 weeks

John R. Hess, Neeta Rugg, Jenny K. Gormas, Amy D. Knapp, Heather R. Hill, Cynthia K. Oliver, Lloyd E. Lippert, Edward B. Silberstein, and Tibor J. Greenwalt

BACKGROUND: Increasing the length of RBC storage can increase both RBC availability and quality. This work addresses 11-week RBC storage in experimental ASs (EASs).

STUDY DESIGN AND METHODS: Three studies were performed. In the first, 24-hour in vivo recovery of ^{51}Cr-labeled autologous RBCs was measured in nine volunteers after storage of their RBCs for 11 weeks in EAS 67. In the second study, 4 units of blood were divided and stored in aliquots with an EAS containing 0, 15, 30, or 45 mmol per L of mannitol; then hemolysis, RBC morphology, and microvesicle protein were measured. In the third study, 6 full units were stored for 12 weeks in the EAS containing 30 mmol per L of mannitol, with weekly sampling for morphologic and biochemical measures of RBC quality.

RESULTS: RBCs stored for 11 weeks in EAS-67 had a mean 24-hour in vivo recovery of 79 ± 5 percent, but the hemolysis was 1.35 ± 0.68 percent. Increasing mannitol content of the EAS reduced hemolysis but increased microvesiculation. EAS-76, with 30 mmol per L of mannitol allowed 11-week storage with 0.48 ± 0.10 percent hemolysis at 11 weeks and 0.62 ± 0.14 percent hemolysis at 12 weeks.

CONCLUSION: It is possible to store RBCs for 11 weeks in EAS with greater than 75 percent recovery and less than 1 percent hemolysis.

Longer RBC storage will require better RBC storage systems.[1,2] The practical limits on RBC storage, hemolysis, and the loss of viability are cumulative events that decrease the quality and utility of the cells. The effects of excessive hemolysis and loss of viability can be partially offset by additional procedures such as cell washing or metabolic rejuvenation. The patients who receive these RBCs and the transfusion medicine specialists who manage these RBCs can all be better served by building improved quality into the next generation of RBC storage solutions and systems.

The authors earlier described 9- and 10-week RBC storage systems using WBC reduction and storage in 200- or 300-mL volumes of hypotonic alkaline experimental ASs (EASs) containing saline, adenine, glucose, mannitol, and disodium phosphate.[3,4] These systems seem to work by supporting an initial increase in RBC ATP synthesis that provides metabolic energy for cell-sustaining processes during longer storage and by suppressing the RBC shape changes and membrane microvesiculation that contribute substantially to hemolysis. The authors also showed that it is possible to achieve greater rates of ATP synthesis during prolonged storage by

ABBREVIATION: EAS(s) = experimental AS(s).

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replacing some of the sodium chloride with sodium bicarbonate.[5]

In an attempt to further increase the time of allowable storage with these SAGMdP EASs, we conducted three studies. First, we conducted a human trial measuring RBC recovery after 11 weeks using bicarbonate-containing EAS-67. This study showed a mean 24-hour RBC recovery of 79 percent, but 1.35 percent hemolysis. Second, we measured the effect of increasing the concentrations of mannitol to reduce hemolysis during 11 weeks of storage. Third, we measured hemolysis during 12 weeks of RBC storage in one of the solutions containing a higher mannitol concentration. This report describes these studies.

MATERIALS AND METHODS

Volunteers

Three groups of healthy volunteers met standard blood donor criteria for allogeneic blood transfusion and individually gave informed consent to participate in these studies.[6] The first (n = 10) and second (n = 4) groups donated full units of blood under protocols approved by the Hoxworth Blood Center and the University of Cincinnati's Institutional Review Board, while the third (n = 6) group donated in accordance with protocols approved by the Institutional Review Board of the Walter Reed Army Institute of Research.

Storage solutions

The compositions of the various EASs[7] are compared in Table 1. The EASs were made in the laboratory from USP adenine, sugars, and salts (Sigma Chemical, St. Louis, MO) and sterilely filtered into 1-L storage bags (4R2032, Baxter Healthcare, Deerfield, IL). The bags were held at 37°C for 2 weeks. The solutions were then cultured and the cultures incubated for another 2 weeks. When sterility was confirmed by the absence of bacteria and fungus growth for 7 to 14 days, the solutions were aliquoted by weight into 600-mL PVC bags (4R2023, Baxter). All connections were made by using a sterile connecting device (SCD 312, Terumo Medical Corp., Elkton, MD).

Study designs

Each of the three studies reported here had a different design, as necessitated by the different natures of the questions asked during each phase of the development process.

TABLE 1. EAS characteristics*

Composition of solutions	EAS-67	EAS-74	EAS-75	EAS-76	EAS-77
NaCl	75	45	45	45	45
$NaHCO_3$	30	30	30	30	30
Na_2HPO_4	9	9	9	9	9
Adenine	2	2	2	2	2
Dextrose	50	50	50	50	50
Mannitol	20	0	15	30	45
pH	8.4	8.4	8.4	8.4	8.4

* Values expressed as mmol per L. All solutions used in 300-mL volume.

In the first study, 10 volunteers each donated a unit of blood. This blood was then processed into packed RBCs and mixed with 300 mL of an EAS containing 30 mEq per L of bicarbonate (EAS-67). The units were sampled and then stored at 1 to 6°C for 11 weeks. At the end of that time, the units were checked for sterility and sampled, and aliquots were radiolabeled with ^{51}Cr and returned to the original donors for the determination of the 24-hour RBC recovery. Samples were analyzed for RBC ATP content and hemolysis.

In the second study, four volunteers each donated a unit of blood. The units were split into four fractions and stored in variants of EAS-67. The variants were designated EAS-74, -75, -76, and -77, and they contained 0, 15, 30, and 45 mmol per L of mannitol, respectively. The units were stored at 1 to 6°C for 11 weeks and sampled at 1 hour and 6 and 11 weeks for biochemical and morphologic measurements.

In the third study, six volunteers each donated a unit of blood, which was stored as packed RBCs in EAS-76 containing 30 mmol per L of mannitol. The units were stored at 1 to 6°C for 12 weeks with weekly sampling for biochemical and morphologic measurements.

RBC unit preparation

In all studies, standard units of blood (450 ± 45 mL) were collected in 63 mL of CPD solution in a triple-bag collection system (4R1402, Baxter). Units were WBC reduced by filtration (Sepacell RS-2000 filters, 4R3303, Baxter). All samples were obtained with a sterile connecting device.

Study 1. Packed RBCs were prepared after centrifugation for 10 minutes as described previously.[7] EAS-67 (300 mL) was added to the units of packed cells by using a sterile connecting device. Units were stored upright at 1 to 6°C for 11 weeks, except for sample removal at 1 week before the recovery studies for fungal and bacterial culture.[8]

Study 2. RBCs were prepared by centrifugation for 10 minutes and divided into four aliquots in 150-mL transfer packs (4C2405, Baxter). Aliquots (75-mL each) of EAS-74, -75, -76, or -77 were added, one each to the RBC aliquots from each original blood donation. Units were stored upright at 1 to 6°C for 11 weeks, except for mixing and sample removal at 1 hour and 6 and 11 weeks.

Study 3. Packed RBCs were prepared by centrifugation for 5 minutes, and 300 mL of EAS-76 was added sterilely. Units were stored upright at 1 to 6°C for 11 weeks, except for weekly mixing and sample removal.

In vitro measurements at the Hoxworth Blood Center

The methods used for the measurement of Hb and MCV, ATP, pH, osmolality and percentage of hemolysis have been described previously.[8] Post-WBC-reduction WBC counts were performed with a Nageotte chamber. The RBC morphology index was measured by a modification of the method of Usry et al.[9] The quantity of total protein shed in microvesicles was determined as described previously.[10]

In vitro measurements at the Walter Reed Army Institute of Research

WBC reduction was confirmed by flow cytometry. The total Hb concentrations were measured with a clinical hematology analyzer (Hematology Cell Counter System Series 9000, Baker, Allentown, PA). Supernatant Hb was measured spectrophotometrically by using the modified Drabkin assay.[11] The percentage of hemolysis was determined by the ratio of free Hb to total Hb. The results are expressed as a percentage of hemolysis to compensate for the differences in Hct and Hb concentrations between samples.

RBC ATP concentrations were measured in the supernatants of deproteinized RBCs. Packed cell aliquots were mixed with cold 10-percent trichloroacetic acid to precipitate blood proteins and centrifuged at 2700 × *g* for 10 minutes, and the protein-free supernatant was frozen at −80°C until tested. ATP was assayed enzymatically by using a commercially available test kit (Procedure 366-UV, Sigma Diagnostics, St. Louis, MO).

Measurement of autologous 24-hour in vivo RBC recovery

On the last day of storage, a 10-mL sample was labeled with approximately 15 μCi of ^{51}Cr as recommended by the International Committee on Standardization in Hematology[12] and Moroff et al.[13] and returned to the donor. Blood samples were drawn at 5, 7.5, 10, 12.5, 15, 20, and 60 minutes and 24 hours after transfusion for survival studies (Cobra II, Packard Instruments, Meriden, CT).

Statistics

Means ± SDs were calculated as descriptive statistics within the study groups. Box plots and line graphs were used to display the data.[14]

RESULTS

Ten units were collected for the 11-week recovery measurements. One broke in handling. The other 9 units were stored for 11 weeks, showed no bacterial or fungal growth, and were labeled and returned to the original donors. The mean 24-hour recovery of RBCs, stored for 11 weeks in 300 mL of EAS-67 and returned to the original donors, was 79 percent when measured by the ^{51}Cr single-label technique (Fig. 1). However, the hemolysis in these units was unacceptably high at 1.35 ± 0.68 percent, which is well above the historic standard of 1 percent for component licensure for routine transfusion. Survival of the cells beyond 24 hours was not measured.

When RBCs were stored in variants of EAS-67 containing increasing concentrations of mannitol, the total hemolysis at the end of 11 weeks of storage declined as the concentration of mannitol increased (Table 2). This suppression of hemolysis with the addition of mannitol occurred in spite of the increasing osmolality, which in turn was associated with less RBC swelling, greater morphologic changes, and greater production of microvesicles. Most of the benefit of adding mannitol for reducing hemolysis was obtained at a concentration of 30 mmol per L.

When 6 additional whole units were stored in the EAS variant with 30 mmol per L of mannitol, EAS-76, hemolysis was 0.48 ± 0.10 percent at 11 weeks and 0.62 ± 0.14 percent at 12 weeks (Fig. 2). The RBC ATP concentrations were maintained above 2.5 μmol per g of Hb throughout the course of 12-week storage.

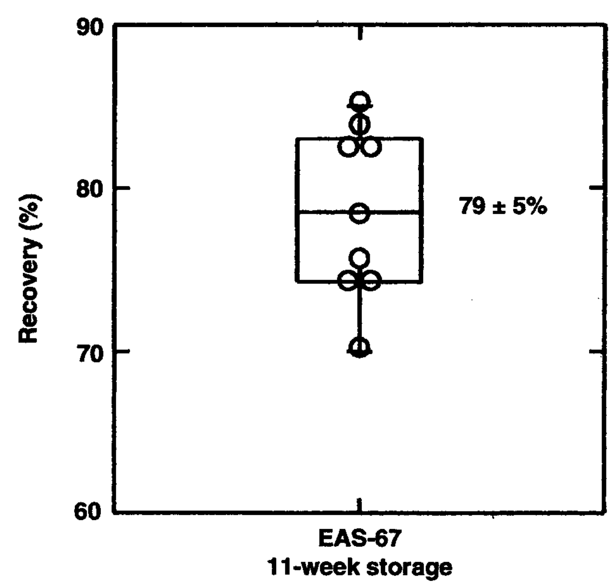

Fig. 1. Twenty-four-hour posttransfusion recovery of RBCs stored for 11 weeks in EAS-67 and labeled with ^{51}Cr (mean ± SD; n = 9).

TABLE 2. Study 2: Solutions and results after 11 weeks of storage*

EAS	Mannitol content (mmol/L)	Osmolality (mOsm/kg H_2O)	MCV (fL)	Morphology index	Vesicle protein (mg/dL RBCs)	Hemolysis (%)	RBC ATP (μmol/g Hb)
EAS-74	0	242 ± 11	112 ± 6	75 ± 6	1.4 ± 0.5	6.69 ± 1.68	4.52 ± 1.54
EAS-75	15	255 ± 12	110 ± 6	68 ± 9	2.6 ± 1.3	1.23 ± 0.39	4.23 ± 0.72
EAS-76	30	271 ± 11	104 ± 4	69 ± 9	3.2 ± 1.8	0.76 ± 0.31	4.48 ± 1.45
EAS-77	45	284 ± 8	98 ± 3	56 ± 8	3.8 ± 1.5	0.71 ± 0.34	4.45 ± 1.40

* Values expressed as mean ? SD; n = 4.

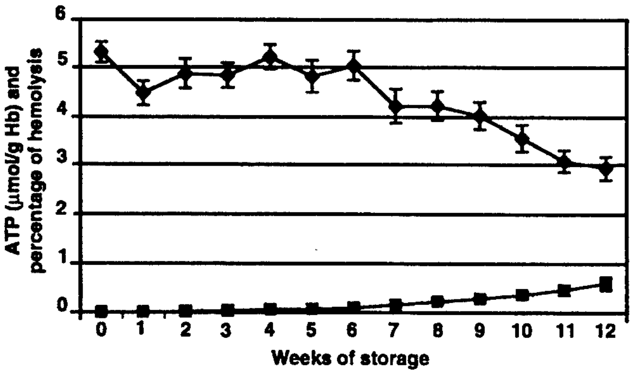

Fig. 2. Weekly measures of RBC ATP concentration (◆) and hemolysis (■) for the RBCs stored in 300 mL of EAS-76 (mean ± SEM; n = 6).

DISCUSSION

We have developed solutions that allow the liquid storage of refrigerated RBCs for 10 weeks. In these solutions, the physical stability of the cells, measured as their fractional hemolysis, and the viability of the cells, measured as their recovery 24 hours after return to the original donor, are as good as those of RBCs from the same donors stored for 6 weeks in presently licensed conventional ASs.[4]

It appears possible to improve the performance of these RBC ASs yet further with simple modifications of the solution composition. Thus, we have shown that it is possible to increase the RBC ATP content by 1 µmol per g of Hb at the end of 11 weeks of storage by replacing 30 mmol per L of sodium chloride with 30 mmol per L of sodium bicarbonate.[5] RBC ATP content is a correlate of RBC viability, suggesting that the viability of RBCs stored in the new solution, EAS-67, might be greater or that such RBCs could be stored longer.

We tested this hypothesis in the first study reported here. WBC-reduced RBCs stored in 300 mL of EAS-67 had a mean recovery of 79 ± 5 percent. However, the hemolysis, which had averaged 0.7 percent in the initial in vitro study, averaged 1.4 percent at the end of this in vivo trial.[5]

To reduce this rate of hemolysis, we added more mannitol to the solution. We did this first in a stepwise manner (Study 2), which showed that increasing amounts of mannitol reduced hemolysis. This occurred despite the simultaneous finding that adding more mannitol increased the osmolality of the solution, which in turn was associated with greater RBC shape change and more membrane loss by microvesiculation. Most of the benefit of adding more mannitol appeared to be achieved at a concentration of 30 mmol per L.

In the final study, a variant of EAS-67 with 30 rather than 20 mmol per L of mannitol, EAS-76, was tested during 12 weeks of in vitro storage. Mean hemolysis was 0.48 ± 0.10 percent at 11 weeks and 0.62 ± 0.14 percent at 12 weeks.

These studies suggest that it is possible to make an 11-week RBC storage solution that would meet the usual FDA criteria of greater than 75 percent RBC recovery and less than 1 percent hemolysis. EAS-76 appears to be such a solution. This solution can probably be improved even further by reducing the osmolality while maintaining the high mannitol concentration. This modification would be expected to have the effect of reducing the increased shape change and microvesiculation to the levels observed with EAS-74. Because the microvesiculation is both an energy-requiring process and a component of the total hemolysis, suppressing it would be expected to increase the RBC ATP content and reduce total hemolysis further.[15-17]

Maintenance of RBC ATP concentrations is a necessary but not always sufficient condition for high RBC recoveries after long-term storage. The correlation of ATP content and recovery is often weak. Thus, additional in vivo studies using larger numbers of volunteers, second radioactive labels for measures of RBC mass independent of stored-cell dilution, and multiple populations would all be helpful. Multiple populations are especially important, because interpersonal differences in the "storability" of RBCs are the largest source of variation in most RBC recovery studies. Not every group will necessarily have the characteristics of the group of white female blood center workers who were our study population. Also, studies of the in vivo survival of such cells after the first 24 hours and of their ability to regenerate 2,3-DPG in vitro would provide additional reassurance that RBCs stored for these long periods might behave normally when returned to the donor.

The utility of increasing liquid RBC storage from 10 weeks to 11 or perhaps 12 weeks is probably small in most clinical settings. However, for the military, which maintains blood in remote locations, for certain autologous uses, and for avoiding seasonal RBC shortages, the benefit may be higher. Moreover, if these storage systems work by improving the overall quality of RBCs throughout their storage life, then most RBC transfusion recipients may benefit.

ACKNOWLEDGMENTS

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Appendix F - (Maintaining RBC ATP for 12 weeks)

•• 327

STORAGE OF PLATELETS IN COMPOSOL-PS ADDITIVE SOLUTION

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Objective/design: Extending the platelet storage period from 5 to 7 days is attractive, as it considerably reduces outdating. However, when additive solutions such as PAS-II are used, up to 18% of the leukoreduced platelet concentrates (LR-PCs) had a pH<6.8 already at day 5. Therefore, the aim of this study was to evaluate the storage of LR-PCs in a new additive solution (Composol-PS) for at least 7 days.
Materials and methods: For one LR-PC, 5 buffy coats (50 mL, hematocrit 40%) were pooled and 250 g Composol-PS (Fresenius, containing saline, citrate, acetate, potassium, magnesium and gluconate) was added. After centrifugation, platelet rich supernatant was expressed through a filter to a 1.3-L butyryl-trihexyl-citrate-plasticised PVC storage container. LR-PCs were stored on a flatbed shaker, and sampled on day 1, 6 and 8. Cell counts were done on an automated counter, and blood gases were measured with a blood gas analyser. We required that $pH_{37°C}$ remained >6.8 and <7.4 throughout storage.
Results: See table (mean ± SD, n=50 LR-PCs in Composol).

		Day 1	Day 6	Day 8
pH		7.00 ± 0.03	7.04 ± 0.12	7.00 ± 0.10
Swirl		2.5 ± 0.6	3.0 ± 0.2	3.0 ± 0.2
pCO_2	mmHg	38 ± 3	30 ± 5	31 ± 5
pO_2	mmHg	76 ± 22	49 ± 21	54 ± 22

On day 1, the LR-PCs contained on average 321±52 $x10^9$ platelets in a volume of 310±7 mL. On day 6 and 8, 2/50 PCs (4%) had pH<6.8, which was associated with a high platelet concentration >1.4 $x10^9$/L. Oxygenation and expelling of carbon dioxide remained adequate up to day 8. The swirling phenomenon remained present.
Conclusion: Composol-PS allows at least 8 day storage with maintenance of pH>6.8, provided the platelet concentration is below 1.4 $x10^9$/L.

•• 328

MAINTAINING RBC ATP FOR 12 WEEKS

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Objective: Eleven week storage of RBC with 79% 24-hour 51-chromium recovery but >1% hemolysis has been reported. The present objective was to develop an additive solution (AS) which will maintain even higher concentrations of RBC ATP with <1% hemolysis.
Design: In vitro study of RBC metabolism and hemolysis during 12 weeks of storage using pooling and weekly sampling to achieve high data resolution.
Materials and Methods: Twenty-four units of leukoreduced CPD RBC were pooled in ABO identical groups of four, mixed and realiquoted as four groups of test units. 300 mL of the experimental ASs containing in mM adenine 2, glucose 50, mannitol 30, disodium phosphate 9, sodium bicarbonate 30, and sodium chloride 30, 35, 40, or 45 at pH = 8.5 were added to the test RBCs. Weekly samples were taken to measure ATP, 2,3-diphosphoglycerate, glucose, lactate, pH, electrolytes, blood gases and morphology. Shed microvesicles were measured at the end of 12 weeks.
Results: In the AS with 30 mM sodium chloride RBC ATP concentrations were maintained above 3 micromol/gHb for 12 weeks, hemolysis was 0.6% and total microvesicle protein, 6 mg/dL RBC. 2,3-DPG was maintained at normal concentrations for 2 weeks.
Conclusion: Optimizing RBC additive solution electrolyte composition can be used to maintain high ATP concentrations and low hemolysis for as long as 12 weeks with 2 week preservation of 2,3-DPG. In vivo studies with this solution are planned.
Supported by the U.S. Army Medical Research and Materiel Command

•• 329

INTERCEPT PLATELETS ARE HEMOSTATICALLY AS EFFECTIVE AS CONVENTIONAL PLATELETS IN THE PROPHYLAXIS AND TREATMENT OF BLEEDING: RESULTS OF THE *SPRINT* TRIAL

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Objective: The INTERCEPT platelet system using Helinx™ technology (amotosalen HCl [S-59] plus UVA light) inactivates a broad spectrum of viruses, bacteria, protozoa, and leukocytes in platelet concentrates. A study evaluated the hemostatic efficacy of apheresis INTERCEPT-treated Platelets (IP) compared to conventional apheresis platelets (CP) in treating severely thrombocytopenic patients (pts) for up to 28 days. Design: *SPRINT* was a randomized, double-blind, controlled, non-inferiority Phase III trial conducted at 12 US centers. Pts received prophylactic platelet (plt) transfusions (PTx) for plt counts below a predefined threshold (usually <10 or <20X10^9/L) and therapeutic plt Tx (TTx) for active bleeding or in preparation for invasive procedures. Methods: Hemostasis evaluation used WHO bleeding criteria, grades 0 (none) to 4 (maximum). Eight organ systems were assessed daily, and for 12 hours preceding and following each Tx. Overall daily bleeding grade was determined for each pt as the highest WHO grade for any organ system.

Results	IP	CP	Pvalue
Number patients	318	327	-
Number Tx	2678	2041	-
Mean days plt support	11.8	10.6	0.08
TTx (% of total Tx)	6.5	9.9	<0.01
Mean PTx per pt	7.9	5.6	<0.01
Mean TTx per pt	0.6	0.6	0.55
Pre-TTx bleeding grade: 1/2/ combined 3 & 4 (%)	22/44/8	18/36/18	0.27
Post-TTx bleeding grade improvement (%)	42	41	0.87

The majority of Tx were prophylactic. Of the TTx, an equal number were given for active bleeding and prior to invasive procedures. The CP group received more therapeutic transfusions than did the IP group. Pre-Tx plt count was 12X10^9/L higher for TTx than for PTx (26X10^9/L vs. 14X10^9/L, respectively) in both groups. Bleeding improved by ≥1 grade following 35% of PTx and 41% of TTx, regardless of treatment assignment. Conclusions: INTERCEPT-treated platelets are hemostatically as effective as conventional platelets in preventing and treating bleeding.

Appendix G - (Report on 12 week storage of red cells in a low chloride)

•• 347

REPORT ON 12 WEEK STORAGE OF RED BLOOD CELLS IN A LOW CHLORIDE, ALKALINE, HYPOTONIC ADDITIVE SOLUTION

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Objective: To investigate the effect of replacing sodium chloride with an equivalent amount of sodium bicarbonate in an additive solution (AS) with acceptable in vivo results for nine week red blood cell (RBC) storage. To establish how relative volumes of the AS would perform with RBCs derived from 400 to 500mL of CPD blood. Design: RBCs were stored for 12 weeks in an alkaline, hypotonic AS with no chloride. Materials and Methods: Twenty-four leukoreduced CPD RBC units, pooled in ABO & D-compatible groups of three, were mixed and realiquoted into three groups of eight test units. The equivalent of 200 mL/450mL unit of the AS (containing in mM: adenine 2, glucose 110, mannitol 55, disodium phosphate 12, sodium bicarbonate 26, pH 8.51, mOsm 256/Kg H_2O) was added. Adenosine 5'-triphosphate (ATP), hemolysis, mean corpuscular volume (MCV), pH, glucose, lactate, pCO_2, pO_2, supernatant K, Na, Cl, Ca were measured to 12 weeks, 2,3-diphosphoglycerate (DPG) at three weeks. Results: In all three groups the ATP was maintained at >107% of initial concentration for 8 weeks and was >3.15µmol/g Hb at 12 weeks. At 12 weeks, hemolysis was <0.6%. Extra- and intracellular pH started at .7.4 and decreased to .6.5 after 12 weeks. Notably, DPG held at the initial level for one week, was >10µmol/g Hb after two weeks and .6µmols after three weeks. Glucose, lactate, pCO_2, pO_2 and other measurements behaved predictably. MCVs were >121fL at 1-hr and 105fL after 12 weeks in all the mixtures, and >95% of the cells were still discocytes. Conclusion: This low chloride, alkaline, hypotonic additive solution promises to make possible storage of clinically acceptable RBCs for twelve weeks.
(Supported by US Army MRAA Award No. DAMD17-01-1-002).

•• 348

FIRST DESCRIPTION OF H VARIANTS IN A GREEK FAMILY – SEROLOGICAL AND MOLECULAR ANALYSIS

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The first H deficient variant, the " Bombay " phenotype, was described in 1952, and was defined by a total lack of ABH activity in erythrocytes and secretions. Its genetic background is due to alterations of the corresponding gene FUT1, which encodes for the L-fucosyltransferase enzyme.
We present the first family with Bombay and para-Bombay phenotype in the Greek population (Caucasians). The determination has been done by use of standard serological methods of ABO and molecular analysis of the FUT1 gene for its genetic alterations.
Serologically we used anti-A, anti-B, anti-AB, anti-D, anti-H tube test and gel test of DIAMED. Conformation tests were performed by the use of ABO known red cells.
We observed anti-A/B/H antibodies (strong) in sera of the members with Bombay phenotype with negative autocontrol (O+, non-secretors). Anti-A and anti-H antibodies (weak) were found in one member of this family with para-Bombay phenotype (B+, secretor) and anti-A, anti-B and anti-H antibodies (weak) in another member with para-Bombay phenotype (O+, secretor).
We isolated genomic DNA from blood from 6 members of the family (DNAzol technique–MRC) and amplified the entire coding region of the FUT1 gene by standard PCR methodology. The amplified fragments (1200 bp) were subsequently fully sequenced by an ABI-310 genetic analyzer. The methodology included 8 primers (forward and reverse). The sequencing revealed two point mutations : 538 C_T/180Gln _stop and 826C_T/276Gln _stop, with an additional silent polymorphism (1088 T_C). The first point mutation and the polymorphism are newly described. This results in a disruption of the transcription of FUT1 gene in the members of the family with Bombay phenotype and a diminished transcription in the members of the family with para-Bombay phenotype.
In conclusion , this is the first Bombay/para-Bombay family case in the Greek population with genetic alterations not previously described. Further investigation will include all members of the family.

•• 349

BUFFERED WASHING IMPROVES RED CELL POST THAW STORAGE

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Objective: Liquid red cell storage is improved when the storage solution pH is increased. This research was undertaken to determine if buffering the deglycerolization wash solution would improve the storage characteristics of deglycerolized blood. Design/Materials and Methods: Twenty-four units were collected into CP2D, leukoreduced, packed to 75% hematocrit and pooled in sets of four of identical ABO. Pooled cells were divided into four aliquots and 100 mL AS-3 added to each aliquot before 4C storage. On the sixth day of storage, the cells were glycerolized in a closed system using the Haemonetics ACP™ 215, and frozen for at least 24 hours. Thawed cells were deglycerolized, two of each set with the unmodified saline dextrose wash, pH 5.5, and two with disodium phosphate (20mmol/L) buffered saline dextrose wash, pH 8.8. One each of the standard and modified wash pairs was stored in AS-3 and the other in an experimental solution (data not shown). Hemolysis and ATP were measured at beginning of post-deglycerolized storage and weekly for 42 days.
Results:

Saline Dextrose	Percent	Hemolysis					ATP % Initial
Solution	Day 7	Day14	Day 21	Day 28	Day 35	Day 42	Day 42
Unbuffered (AS-3)	0.2	0.3	0.5	0.6	0.8	1.0	27
Buffered (AS-3)	0.2	0.3	0.5	0.6	0.7	0.8	61

Conclusion: Liquid blood stored in AS-3 for 42 days has been previously reported having 0.9% hemolysis and 59% of initial ATP. In this study, hemolysis with AS-3 resuspended cells was comparable to liquid stored AS-3 blood, while buffering the saline/dextrose deglycerolization solution maintained ATP levels better than non-buffered solution. Therefore, hemolysis and ATP

Appendix H - (The effects of phosphate, pH, and additive solution volume on RBCs stored in saline-adenine-hposphate-glucose-mannitol additive solutions)

The effects of phosphate, pH, and AS volume on RBCs stored in saline-adenine-glucose-mannitol solutions

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BACKGROUND: RBC ATP concentrations are the most important correlate of RBC viability. Tests were performed to determine whether increased AS volume, pH, and phosphate content increased stored RBC ATP concentrations.

STUDY DESIGN AND METHODS: In three studies, packed RBCs were pooled in groups of 3 or 4 units and realiquoted as combined units to reduce intradonor differences. Pooled units were stored in the licensed ASs, AS-1 or AS-5, which contain saline, adenine, glucose, and mannitol (SAGM), or in experimental ASs (EASs) containing SAGM and disodium phosphate. Ten pools were stored in AS-1 at RBC concentrations equivalent to 100, 200, or 300 mL of AS. Six pools were stored in 100, 200, 300, or 400 mL volumes of EAS-61. Ten pools were stored in 100 mL of AS-5, 200 mL of EAS-61, or 300 mL of EAS-64. RBC ATP concentration and other measures of RBC metabolism and function were measured weekly.

RESULTS: RBC ATP concentrations decreased sooner with storage in increasing volumes of AS-1. In EAS-61 and EAS-64, RBC ATP concentrations initially increased and stayed elevated longer with increasing AS volume.

CONCLUSIONS: The addition of disodium phosphate to SAGM AS increases the RBC ATP concentrations. Reducing storage Hct appears to have a separate beneficial effect in reducing hemolysis.

RBC storage systems with longer outdating will have many beneficial effects.[1] The burden of nonviable RBCs will be reduced for most transfusion recipients. Some of the several hundred thousand RBCs that now outdate each year will not be lost, and the ability to maintain adequate emergency stocks in remote locations will be enhanced. Autologous transfusion schemes will function better if RBCs can be stored longer, have better viability, and contain fewer products of cell degradation.

The development of better RBC storage systems has been slow. For the last decade, 6-week RBC storage has been the United States standard. That standard is based on the use of 100-mL of AS containing either saline, adenine, glucose, and mannitol (SAGM, as in AS-1 and AS-5) or saline, adenine, glucose, and monosodium phosphate (SAGP, as in AS-3).[2] Two 7-week RBC storage systems have been licensed in Europe,[3,4] but the 24-hour viability of cells stored in these solutions is barely 75 percent. The promise of 14-week RBC

ABBREVIATIONS: EAS(s) = experimental AS(s); SAGM = saline-adenine-glucose-mannitol.

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DISCLOSURE: The authors certify that they are employed, directly or indirectly, by the U.S. Army and the University of Cincinnati, which have jointly filed for patent rights on EAS-61 and EAS-64. Two of the authors (JRH and TJG) are listed as inventors on the patent filing.

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demonstrate improved viability with the storage of rabbit RBCs in Solution 6. Finally, in 1996, Dumaswala and colleagues[9] reported a comparison of two hypotonic media, one with phosphate and one without. The less hypotonic medium with and a higher initial pH had better preservation of RBC ATP concentrations.

The demonstration of 14-week RBC storage by Meryman et al. has always been clouded by the recognition that, "[f]rom the clinical standpoint, Solution number 6 would clearly be unacceptable for transfusion...."[2(p505)] Attempts to understand and translate this work into clinically useful storage solutions have been limited by the failure to measure clinically relevant endpoints, such as human RBC viability and storage hemolysis, with reasonable statistical power. Future work in this field should attempt to maximize RBC ATP concentrations by the manipulation of pH, adenine, and phosphate content and to minimize hemolysis by WBC-reduction. Progress can be made with candidate ASs and storage systems incorporating these innovations.

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storage heralded in a 1986 article by Meryman et al.[5] has not been realized in transfusable solutions. Nevertheless, the fact that long-term viability is possible in solutions that drive ATP synthesis suggests the most likely path for future development.

We report here the results of three pooling studies that measured RBC ATP concentrations with increasing volumes of the ASs. The experimental ASs (EASs) with greater phosphate, pH, and volume increased RBC ATP concentrations, whereas the traditional SAGM solutions led to decreased RBC ATP concentrations at greater dilutions. These studies were performed in two separate laboratories. They are reported together because they help explain why present storage solutions limit viability to 6 weeks and how transfusable solutions that will extend RBC viability beyond that limit might be formulated.

MATERIALS AND METHODS

Volunteers

Two groups of 30 healthy volunteers, who were donating full units of blood for the collection of anthrax-immune plasma, consented to the use of their RBCs for Studies 1 and 3. The donors met standard blood donor criteria and gave informed consent in accordance with protocols approved by the Institutional Review Board of the Walter Reed Army Institute of Research and the US Army's Human Subjects Research Review Board. Volunteers were tested to exclude sickle Hb trait (Sicklescreen, Pacific Hemostasis, Huntersville, NC).

In Study 2, 24 volunteers donated full units of blood under protocols approved by the Hoxworth Blood Center and the University of Cincinnati's Institutional Review Board.

Storage solutions

The compositions of AS-1 (Adsol, Baxter Healthcare Corp., Roundlake, IL), AS-5 (Optisol, Terumo Medical Corp., Somerset, NJ), EAS-61, and EAS-64 are compared in Table 1. The significant differences in the solutions are the reduced salt content and the presence of disodium phosphate in the EASs. The presence of disodium phosphate raises the pH of the EASs to about 8.5 at room temperature, whereas the pH of AS-1 and AS-5 is 5.5.

The EASs were made in the laboratory from high-purity adenine, sugars, and salts and were sterilely filtered into 1-L storage bags. When sterility was confirmed by the absence of bacterial and fungal growth for 7 to 14 days, the solutions were aliquoted by weight into 600-mL bags. All connections were made by using a sterile connecting device (SCD 312, Terumo Medical Corp., Elkton, MD).

Study design

We conducted three pooling studies to evaluate RBC metabolism and physiology over the period of storage. Pooling reduces the largest source of variability in conventional blood storage studies—the differences between the RBCs from different donors—by placing some of the cells from every donor in every arm of the study while maintaining conventional unit size and geometry. In each of the three studies, the RBC units were grouped into sets of 3 or 4 ABO-matched units; each set was then pooled, mixed, and realiquoted into identical pooled units. For example, in Study 1, 30 RBC units were collected and grouped into 10 sets of 3 ABO-matched units. Each set of 3 matched units was then pooled, mixed, and realiquoted as 3 identical pooled units. Ten pooled units, 1 from each set, were used in each arm of the study.

TABLE 1. Composition of ASs used in this study (concentrations in m*M*)

	CPD	AS-1	AS-5	EAS-61	EAS-64
Dextrose	142	111	45	110	50
Na_3 citrate	104				
Adenine		2	2.2	2	2
NaCl		154	150	26	75
Mannitol		41	45.5	55	20
Citric acid	18				
Na_2HPO_4				12	9
pH at room temperature	5.5	5.5	5.5	8.2	8.5

In Study 1, units were stored in AS-1 at RBC concentrations equivalent to dilution in 100, 200, and 300 mL of AS. In Study 2, units were stored in 100-, 200-, 300-, or 400-mL volumes of EAS-61. In Study 3, units were stored in 100 mL of AS-5, 200 mL of EAS-61, or 300 mL of EAS-64. Electrolytes, pH, metabolites, RBC morphology scores, and RBC ATP concentrations were measured weekly during storage by sterile sampling techniques.

RBC unit preparation

Study 1, AS-1 units. Standard units (450 ± 45 mL) of blood were collected from each donor into 63 mL of CPD in the primary PVC bag of an AS-1 triple-bag collection set (code 4R-1436, Baxter). Packed cells were prepared by centrifugation at 5000 × *g* for 5 minutes at room temperature, which was followed by the removal of sufficient plasma to achieve a Hct of 80 to 85 percent. ABO-matched packed RBCs were then pooled in groups of 3 units in 1-L sterile bags (Code 4R-20-32, Baxter), mixed thoroughly, and aliquoted into the study units (by weight) using a sterile connecting device for all the transfers. The study units were prepared in 600-mL transfer bags (Code 4R-2023, Baxter) by mixture of the packed RBC aliquot with sufficient AS to produce final storage Hcts of 55, 43, and 35 percent. The result is essentially equivalent to the addition of 100, 200, or 300 mL of AS, respectively, to 200 mL of RBCs.

Study 2, EAS-61 units. Standard units (450 ± 45 mL) of blood were collected from each donor into 63 mL of CPD in the primary PVC bag of an AS-1 triple-bag collection set (Code 4R-1436). Packed cells were prepared by centrifugation at 5000 × *g* for 5 minutes at room temperature, which was followed by the removal of plasma to achieve a target Hct of 80 percent. Packed RBCs were pooled, as above, in sets of 4 units and realiquoted. Finally, EAS-61 was added in amounts of 100, 200, 300, and 400 mL to achieve target storage Hcts of about 66, 55, 46, and 40 percent, respectively. The storage Hcts are higher with EAS-61 than with AS-1 because the hypotonic EAS leads to a 20-percent swelling of the RBCs with an equivalent increase in the cellular fractional volume.

Study 3, AS-5, EAS-61, and EAS-64 units. Standard units (450 ± 45 mL) of blood were collected from each donor into 63 mL of CPD in the primary PVC bag of an AS-5 double-bag collection set (Code 1BB*AGD456A, Terumo). Packed cells were prepared by centrifugation at 5000 × *g* for 5 minutes at room temperature, which was followed by the removal of sufficient plasma to achieve a Hct of 80 to 85 percent. Three units of identical ABO type were then pooled and realiquoted as described above, and either 100 mL of AS-5, 200 mL of EAS-61, or 300 mL of EAS-64 was added. All units were gently mixed by inversion 25 times, sampled aseptically for in vitro testing, and placed in refrigerated storage (1-6°C) 4 hours or less after collection.

In vitro measurements

Samples from stored units were collected, after gentle mixing by inversion, by a sterile-sampling procedure in a biologic safety cabinet. A battery of in vitro tests was performed on all units at the beginning of storage and weekly thereafter.

For Studies 1 and 3, performed at Walter Reed, the total Hb concentration, WBC counts, and RBC counts were measured with a clinical hematology analyzer (Hematology Cell Counter System Series 9000, Baker, Allentown, PA). Supernatant Hb was measured spectrophotometrically by using the modified Drabkin's assay.[6] The percentage of hemolysis was determined by the ratio of free Hb to total Hb. The results are expressed as the percentage of hemolysis to compensate for the differences in Hct and Hb concentrations in samples. Centrifuged microHcts (Clay Adams, Becton Dickinson, Rutherford, NJ) were performed to avoid the readjustment of cell volume and subsequent errors caused by the isotonic diluent used in the blood analyzers. Mean corpuscular volume was calculated from the microHct and the RBC count.

RBC ATP concentrations were measured in deproteinized supernatants. Whole-blood or packed cell aliquots were mixed with cold 10-percent trichloroacetic acid to precipitate blood proteins and centrifuged at 2700 × *g* for 10 minutes, and the protein-free supernatant was frozen at −80°C until it was tested. ATP was assayed enzymatically by using a commercially available test kit (Procedure 366-UV, Sigma Diagnostics, St. Louis, MO).

Blood gases and pH were measured on a blood gas analyzer (855, Corning, Ithaca, NY). Thus, pH was measured at 37°C. Phosphate and glucose were measured on a programable chemical analyzer (Cobas Fara, Roche Diagnostic Systems, Nutley, NJ).

For Study 2, performed at the Hoxworth Blood Center, a different clinical hematology analyzer was used (MaxM, Coulter Electronics, Hialeah, FL). The pH was measured with a benchtop pH meter (900A Orion, Research, Boston, MA) at 22°C. Blood pH has a temperature coefficient of −0.015 pH units per degree (C), so pH measured at 22°C will be about 0.22 pH units higher than pH measured at 37°C. Supernatant potassium, glucose, and inorganic phosphorus testing was sent to an outside laboratory (Health Alliance Laboratories, Cincinnati, OH). Inorganic phosphate was measured by using a procedure of Boehringer Mannheim (Mannheim, Germany) that involves the formation of ammonium phosphomolybdate, which was detected in an automated chemistry analyzer (747-200, Boehringer Mannheim Corp., Indianapolis, IN). RBC ATP concentrations were measured as above, except that the RBC suspensions were deproteinized with 12-percent trichloroacetic acid. Supernatant Hb was measured with 3,3´,5,5´-tetramethylbenzidine (Procedure No. 527, Sigma Diagnostics). The average degree of RBC shape change from discocytes to echinocytes to spherocytes was measured as RBC morphology scores on 200 cells, determined according to the method of Usry et al.[7]

Statistical analysis

Comparisons of means of measured values at given times within the individual crossover trials were evaluated with ANOVA. Probabilities less than 0.05 were considered significant.

RESULTS

Study 1. RBCs were stored in increasing volumes of AS-1. RBCs stored in the conventional 100 mL of AS-1 maintained RBC ATP concentrations close to their initial value of 5.2 μmol per g of Hb for 4 weeks (Fig. 1A). Thereafter, the RBC ATP concentration declined by 0.6 μmol per g of Hb per week. With increasing volumes of the AS, the initial RBC ATP concentration rose higher, to 5.5 and 5.8 μmol per g of Hb, but the subsequent decline began sooner, at 2.5 and 1 weeks, respectively. However, the rate of decline remained the same at 0.6 μmol per g of Hb per week.

Supernatant pH was the same at each weekly measurement at all three suspension concentrations (Fig. 1B). Inorganic phosphate, initially from the residual plasma and the CPD and later from the breakdown of ATP and 2,3 DPG, was diluted in the additional volumes of AS-1 (Fig. 1C). The additional AS-1 volume decreased the RBC hemolysis during storage by approximately 50 percent (Fig. 1D).

Study 2. RBCs were stored in increasing volumes of EAS-61. RBC ATP concentrations were different after just 1 hour of storage, averaging 4.11 ± 0.37 μmol per g of Hb with 100 mL of EAS-61, 4.54 ± 0.34 with 200 mL, 4.96 ± 0.15 with 300

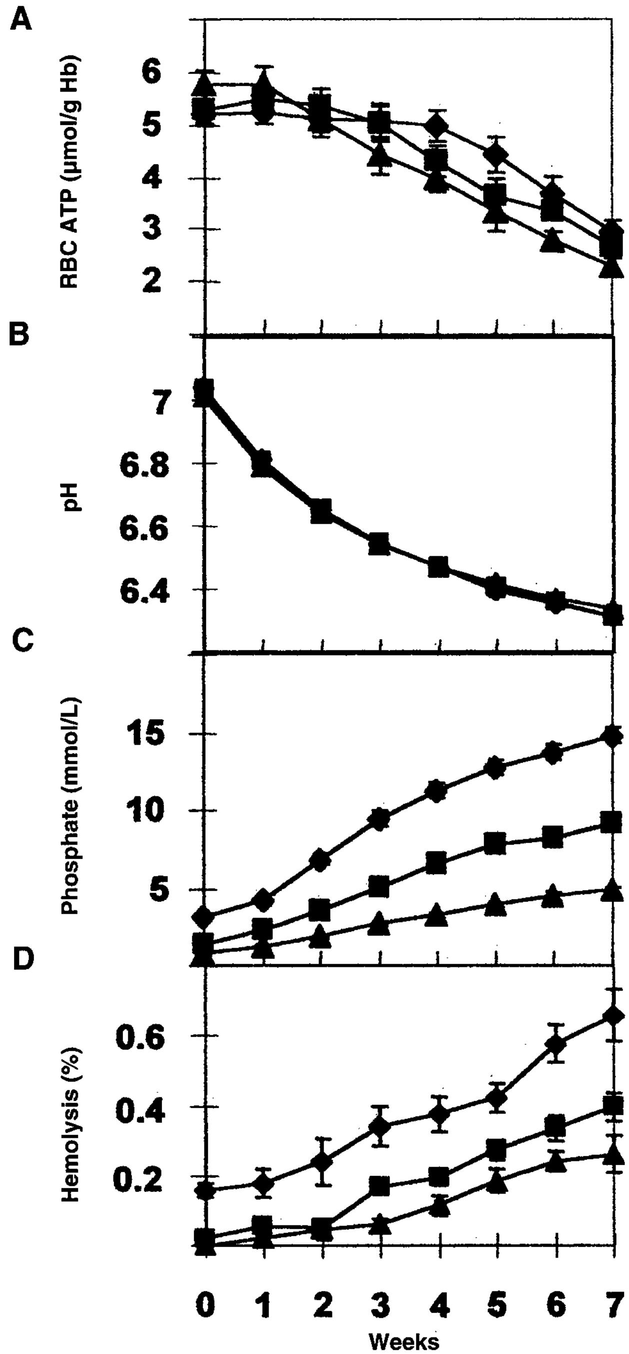

Fig. 1. The RBC ATP concentration (A), supernatant pH measured at 37°C (B), supernatant inorganic phosphate concentration (C), and fractional hemolysis (D) of pooled RBC units stored in the equivalent of 100 (-◆-), 200 (-■-), and 300 (-▲-) mL of AS-1 (n = 10 measurements at each point, data presented as mean ± SEM). The increasing volume of AS-1 led to reduced RBC ATP concentrations in the later phases of storage, despite equivalent pH and higher phosphate concentrations. The increased volume led to improved RBC integrity.

mL, and 5.11 ± 0.25 with 400 mL (Fig. 2A). Over the next 2 weeks, these concentrations increased to 4.38 ± 0.47, 4.88 ± 0.30, 5.34 ± 0.39, and 5.56 ± 0.39 µmol per g of Hb, respectively. After that time, the RBC ATP concentration declined at a uniform rate of about 0.35 µmol per g of Hb per week in all groups.

Supernatant pH was again the same at each weekly measurement (Fig. 2B). Inorganic phosphate concentrations were all approximately 6 mmol per dL (data not shown). The RBC morphology indices were 90- to 100-percent disco-cytes for all groups except for the 100-mL AS volume group (Fig. 2C). The AS volumes of 200 mL or greater were also associated with a 50-percent reduction in RBC hemolysis (Fig. 2D).

Study 3. RBCs were stored in 100 mL of AS-5, 200 mL of EAS-61, and 300 mL of EAS-64. RBC ATP concentrations were initially identical in all groups, about 4.8 ± 0.3 µmol per g of Hb. They remained stable in the AS-5 group for the next 2 weeks, but, in the EASs, they increased by about 10 percent over the first 2 weeks and remained elevated until 5 weeks (Fig. 3A). In AS-5, the RBC ATP concentrations decreased after 2 weeks of storage, at a steady rate of about 0.4 µmol per g of Hb per week. The same rate of decrease was observed with storage in the EASs, but the decrease started at 5 weeks of storage. Thus, the RBC ATP concentrations in the units stored in the EASs were about 1 µmol per g of Hb higher between 5 and 10 weeks of storage, or they remained above any given level for about 2.5 weeks longer.

Supernatant pH decreased more rapidly in the first 2 weeks in the smaller volume of the AS-5 system and less rapidly in the later phases of storage (Fig. 3B). Supernatant phosphate concentrations behaved very differently in the three storage solutions, rising continuously in AS-5, decreasing initially in EAS-61, and remaining relatively constant in EAS-64 (Fig. 3C). Both of the EASs reduced RBC hemolysis by 75 percent (Fig. 3D).

DISCUSSION

In 1986, Meryman et al.[5] showed that RBCs could be stored in experimental nutrient solutions for as long as 14 weeks and could have in vivo 24-hour recoveries greater than 75 percent. Their storage solutions included conventional ingredients such as adenine, phosphate, citrate, and mannitol, but also unconventional ingredients such as ammonium and potassium salts. They also used unconventional formulations with low salt concentrations and neutral pH. The group was not able to discover which of the ingredients were responsible for the marked elevation of RBC ATP concentrations that seemed to be responsible for the prolonged viability.

Five laboratories have confirmed and attempted to extend the work of Meryman's group. In 1990, Greenwalt and colleagues[8] reported splitting units of RBCs and storing them, half in AS-1 and half in Meryman's Solution 6.

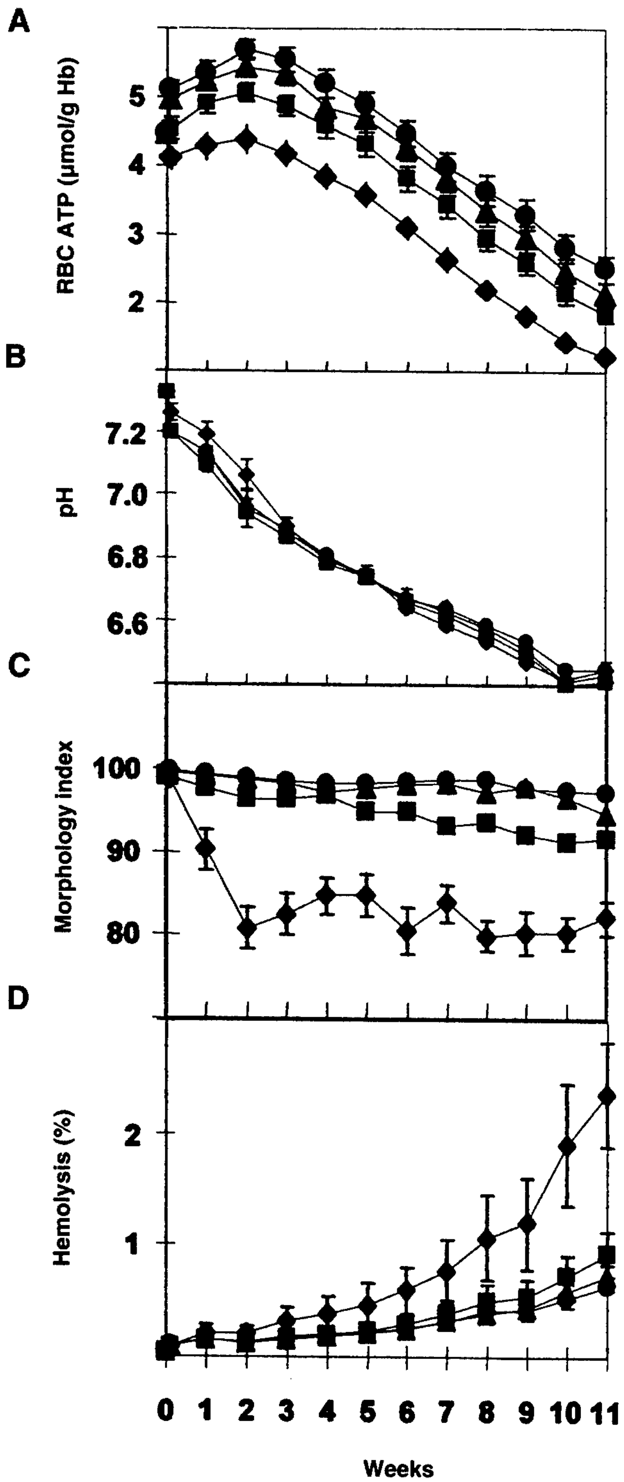

Fig. 2. The RBC ATP concentration (A), supernatant pH measured at 22°C (B),morphology index (C), and fractional hemolysis (D) of pooled RBC units stored in 100 (-◆-), 200 (-■-), 300 (-▲-), and 400 (-●-) mL of EAS-61 (n = 6 measurements at each point, data presented as mean ± SEM). The increasing volume of AS led to increased RBC ATP concentrations in the later phases of storage, despite equivalent pH. The increased volume led to improved morphology indexes and improved RBC integrity.

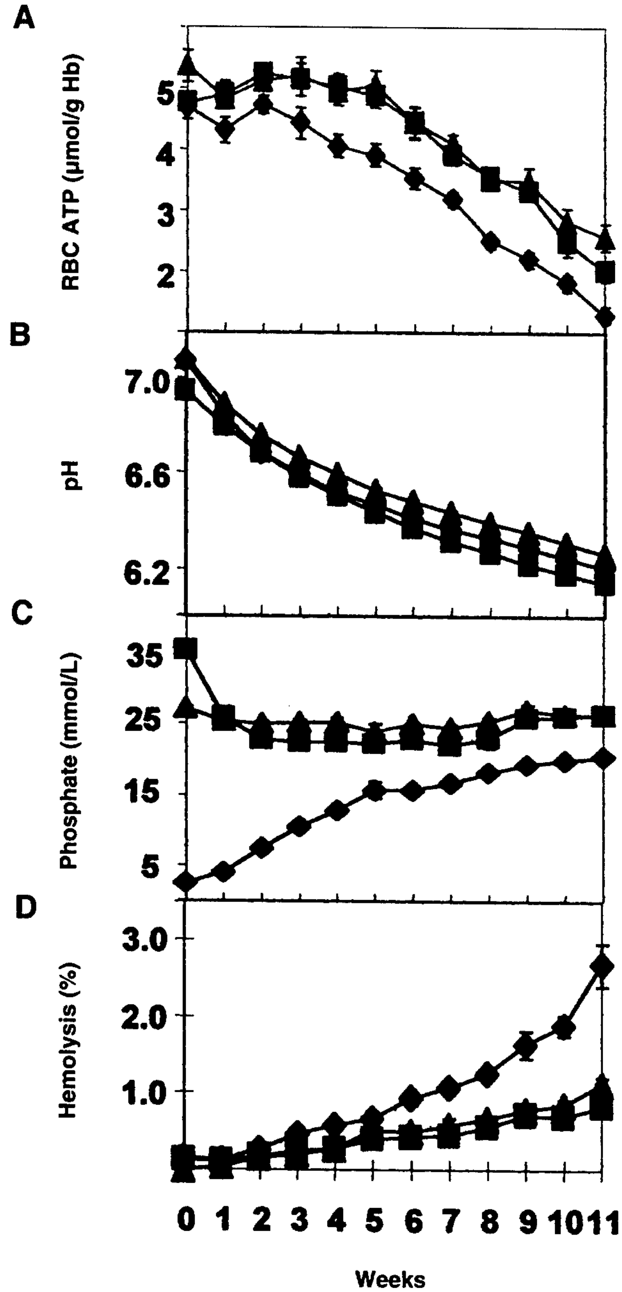

Fig. 3. The RBC ATP concentration (A), supernatant pH measured at 37°C (B), supernatant inorganic phosphate concentration (C), and fractional hemolysis (D) of pooled RBC units stored in 100 mL of AS-5 (-◆-), 200 mL of EAS-61 (-■-), and 300 mL of EAS-64 (-▲-) (n = 10 measurements at each point, data presented as mean ± SEM). The EASs appear to maintain RBC ATP concentrations 2.5 to 3 weeks longer and cause a marked reduction in RBC hemolysis during prolonged storage.

They were able to confirm the increased RBC ATP concentrations in the Meryman solution, but also showed that the low salt solution did not result in better membrane preservation. In 1992, this group reported that increasing amounts of disodium phosphate resulted in successive increases in

RBC ATP concentrations.[9] In 1993, they described acceptable in vivo RBC recoveries after 8 and 9 weeks of storage in 200 mL of an ammonium- and phosphate-containing AS, but they stated that the ammonium would have to be removed at the end of storage.[10] In 1990, Mazor et al.[11] showed that the ammonium and potassium in Meryman Solution 6 were not critical, as they could be replaced with sodium or rubidium, a monovalent cation about the same size as ammonium, with no difference in the initial rise in RBC ATP concentrations. In 1994, Mazor et al.[12] showed that the RBC ATP concentration was most directly affected by the pH and the adenine and phosphate content. In 1992, Kay and Beutler[13] examined the mechanism by which Meryman's Solution 6 increased stored RBC ATP concentrations. They concluded that the inhibition of phosphofructokinase by ATP was blocked by the ammonia in the solutions, which led to higher than normal concentrations of ATP. Also in 1992, Dumaswala and colleagues[14] compared SAGM solutions supplemented with glutamine or glutamine and phosphate. The phosphate-containing solution showed increased RBC ATP concentrations. Finally, in 1993, Högman and collaborators[15] produced a storage solution, in which half-strength citrate in the anticoagulant and a mixture of monosodium and disodium phosphate in the AS led to a higher pH and phosphate content in the final storage solution. When used as a 100-mL AS, this solution allows RBC storage for 7 weeks, and it is now licensed in Europe as ErythroSol (Baxter International, Deerfield, IL).

The data from these studies did not directly address the effects of storage solution volume, but they suggested that it might be possible to make directly infusable, 200-mL, 8- or 9-week RBC storage solutions. Therefore, we undertook to measure directly the effect of AS volume by using two experimental and two conventional RBC storage solutions. In Study 1, increasing volumes of AS-1 resulted in lower RBC ATP concentrations but also in less hemolysis in a volume-dependent manner. In Study 2, increasing volumes of EAS-61 resulted in increased RBC ATP concentrations and reduced hemolysis in a volume-dependent manner. In Study 3, AS-5, an AS-1-like SAGM solution, and EAS-61 were directly compared along with a third 300-mL EAS, EAS-64. Storage in the EASs again resulted in greater RBC ATP concentrations and lower hemolysis during storage from those with conventional volumes of the licensed solution.

The increased phosphate concentration and pH of the EASs appears to lead to increased RBC ATP synthesis and thus to higher RBC ATP concentrations throughout storage. Högman et al.[15] demonstrated this effect in a 7-week storage solution. Increasing the AS volume raised the final concentration of phosphate and adenine in the storage bag and appeared to drive the RBC ATP concentrations even higher. The increased storage solution volume also had a beneficial effect, improving RBC morphology and reducing storage hemolysis. This effect of increased AS volume has not previously been recognized. The effect is important, for if EAS-61 were used as an AS at a 100-mL volume, it would not meet the current FDA storage solution licensing requirement of less than 1 percent hemolysis during storage beyond about 7 weeks.[16] Moreover, the improvement in storage hemolysis observed with increasing volumes of EAS-61 solution appears to be greater than that observed with AS-1. This suggests that the reduced hemolysis is an effect of both the increased storage volume and the better maintenance of RBC energy metabolism. Hypotonic swelling of the cells does not appear to be critical for this volume-related reduction in hemolysis, because the beneficial effect was observed with storage in increasing volumes of isotonic AS-1. Thus, it is not obvious from the data why storage solution volume is important. The cells, although mixed weekly for sampling, spend most of their time at very high local concentrations in the bottom of the storage bag.

EAS-61 and EAS-64 are directly infusable RBC storage solutions composed of saline, adenine, dextrose, mannitol, and disodium phosphate in acceptable concentrations. These materials are already used in approved RBC storage solutions in the United States and/or Europe. The downside of pooling studies, such as the ones described here, is that, after the blood is pooled, it cannot be returned to the original donor in studies of RBC recovery and survival. Only the surrogates of recovery—RBC ATP concentration and RBC morphology—can be measured in such studies. However, the data presented here do suggest that EAS-61 and EAS-64 should be clinically tested to determine the adequacy of 24-hour in vivo RBC recovery after storage for 7, 8, 9, and 10 weeks. Longer-lasting and better RBC storage will reduce RBC losses, improve the availability of blood in remote locations, make autologous transfusion programs more practical, and improve transfusion safety.

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Appendix I - (The role of Electrolytes and pH in RBC additive solutions)

The role of electrolytes and pH in RBC ASs

John R. Hess, Neeta Rugg, Amy D. Knapp, Jennifer F. Gormas, Heather R. Hill, Cindy K. Oliver, Lloyd E. Lippert, and Tibor J. Greenwalt

BACKGROUND: Experimental additive solutions (EASs) containing saline, adenine, glucose, mannitol and disodium phosphate can support RBCs for 9 or 10 weeks if used in 200- or 300-mL volumes. The effects of variations in the electrolyte composition and volume of EASs were explored.

STUDY DESIGN AND METHODS: In three four-arm studies, 24 RBC units were pooled in groups of 4 and realiquoted as test units to ensure that all donors were equally represented in each study arm. In Study 1, units were stored for 11 weeks in EAS containing 0, 10, 20, or 30 mmol per L of sodium bicarbonate. In Study 2, units were stored for 9 weeks in EAS containing 26, 50, 100, or 150 mmol per L of sodium chloride. In Study 3, units were stored in 100 or 200 mL of AS-3 or EAS-61. RBC ATP concentrations and hemolysis were measured weekly.

RESULTS: Increasing the sodium bicarbonate content of EASs increased the pH throughout storage and increased RBC ATP concentrations in the later phases of storage, but it had no effect on hemolysis. Increased sodium chloride content of EASs led to lower RBC ATP concentrations and increased hemolysis. In EAS-61, RBC ATP concentrations were increased throughout storage, and hemolysis was lower than that of RBCs stored in AS-3.

CONCLUSION: RBC ATP synthesis is highly dependent on the pH of the AS. Hemolysis is affected by the salt content and volume of the AS.

Better RBC storage solutions will improve RBC availability and safety.[1] With the current 5- and 6-week liquid storage systems, 600,000 RBC units outdate each year across the United States. As many as half of these outdating units probably could find a recipient if storage times were 2 weeks longer. Remote centers and hospitals would find it easier to maintain blood inventories to meet emergency needs if longer storage times allowed stock rotation with regional blood centers. Autologous blood storage would also be more useful if liquid storage times were longer. This would allow such donors both to give multiple units and to enter the operating room with a normal Hb. Greater use of autologous blood would free allogeneic RBCs for other uses.

Conventionally stored RBCs show a storage lesion that includes the loss of membrane, ATP, 2,3 DPG, and viability.[2] These RBC changes may injure the transfusion recipient.[3] Storage-related loss of membrane makes RBCs rigid, reducing microvascular flow.[4] Shed RBC membrane microvesicles in the stored RBC supernatant are procoagulant, and lysophospholipids, such as platelet-activating factor, shed from RBC membranes during storage, are proinflamatory.[5-7] Loss of RBC ATP during storage reduces the amount available for secretion, a mechanism by which RBCs control microvas-

ABBREVIATION: EAS(s) = experimental AS(s).

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cular tone.[8,9] Loss of 2,3 DPG increases RBC oxygen affinity and can reduce peripheral oxygen delivery.[10] The administration of nonviable RBCs can lead to a partial blockade of macrophage clearance mechanisms and can reduce the clearance of bacteria in patients with injury or infection.[11] All of these problems may be diminished with better RBC ASs.

We have developed 9- and 10-week RBC storage systems that appear to decrease most of the above-mentioned components of the storage lesion.[12,13] The systems are based on storing packed RBCs in 200 or 300 mL of experimental ASs (EASs) composed of saline, adenine, glucose, mannitol, and disodium phosphate at pH 8.4. They work best in combination with WBC reduction.

This work describes three pooling studies that further explored the effects of the pH and the electrolyte composition of these EASs on storage metabolism, shape change, and hemolysis of the RBCs. In each study, 24 units of packed RBCs were pooled in groups of 4 units and aliquoted into mixed units of conventional size. Within each study, 1 unit from each pool was allocated to each of the four arms. Thus, blood from each of the 24 donors was equally represented in each arm of each particular study. In the first study, the effect of adding sodium bicarbonate to the storage media in concentrations of 0, 10, 20, and 30 mmol per L was examined. In the second study, the effect of varying the sodium chloride concentration was explored with concentrations of 26, 50, 100, and 150 mmol per L. Finally, the combined effects of saline, phosphate, and pH were explored in a comparison of the storage of whole units of packed RBCs in 100 or 200 mL of AS-3 and that in 100 or 200 mL of EAS-61. The results suggest that a high pH is important for ATP synthesis throughout storage and that a low salt concentration reduces hemolysis by decreasing microvesiculation. Phosphate, reported to drive ATP synthesis, had a much smaller effect than pH in the ranges found in these solutions.

MATERIALS AND METHODS

Volunteers

Three groups of 24 healthy volunteers met standard blood donor criteria and gave informed consent to participate in these studies. The first two groups donated full units of blood under protocols approved by the Hoxworth Blood Center and the University of Cincinnati Institutional Review Board, while the third group donated in accordance with protocols approved by the Institutional Review Board of the Walter Reed Army Institute of Research.

Storage solutions

The compositions of the various EASs and AS-3 (Nutricel, Pall Medical, Covina, CA) are compared in Table 1. The EASs were made in the laboratory from high-purity adenine, sugars, and salts and sterilely filtered into 1-L storage bags (Code 4R2032, Baxter Healthcare, Deerfield, IL). The solutions were then cultured and the cultures incubated for 2 weeks. When sterility was confirmed by the absence of bacterial and fungal growth for 14 days, the solutions were aliquoted by weight into 600-mL bags (Code 4R2023, Baxter). All connections were made by using a sterile connecting device (SCD 312, Terumo Medical Corp., Elkton, MD).

Study design

We conducted three pooling studies to evaluate RBC metabolism, morphology, and hemolysis over the course of storage. Pooling reduces the largest source of variability in conventional blood-storage studies, the differences between RBCs from different donors, by placing some of the cells from every donor in each arm of the study while maintaining conventional unit size and geometry. In each of the three studies, the RBC units were grouped into sets of 4 ABO-matched units, and each set was then pooled, mixed, and realiquoted into identical pooled units. Six pooled units, 1 from each set, were used in each arm of each study.

In the first study, units were stored in variants of EAS-64 with increasing sodium bicarbonate concentrations but a constant total sodium concentration and a constant 300-mL AS volume. In the second study, units were stored in variants of EAS-61 with increasing sodium chloride concentrations but at a constant 200-mL volume. In the third study, units were stored in 100 and 200 mL of AS-3 and in 100 and 200 mL of EAS-61. Electrolytes, pH, metabolites, RBC morphology

TABLE 1. Composition of the ASs*

	Study 1				Study 2				Study 3	
	EAS-64	EAS-65	EAS-66	EAS-67	EAS-61	EAS-71	EAS-72	EAS-73	AS-3	EAS-61
Volume (mL)	300	300	300	300	200	200	200	200	100 and 200	100 and 200
NaCl	75	65	55	45	26	50	100	150	70	26
$NaHCO_3$	0	10	20	30	0	0	0	0	0	0
NaH_2PO_4	0	0	0	0	0	0	0	0	23	0
Na_2HPO_4	9	9	9	9	12	12	12	12	0	12
Adenine	2	2	2	2	2	2	2	2	2	2
Dextrose	50	50	50	50	110	110	110	110	55	110
Mannitol	20	20	20	20	50	50	50	50	0	50
Citric acid									2	
$Na_3Citrate$									23	
pH	8.4	8.4	8.4	8.4	8.4	8.4	8.4	8.4	5.8	8.4

* All concentrations in mmol per L.

indices, and RBC ATP concentrations were measured weekly during storage by sterile sampling techniques.

RBC unit preparation

Study 1: EAS-64 variant units. Standard units (450 ± 45 mL) of blood were collected from each donor into 63 mL of CPD in the primary PVC bag of an AS-1 triple-bag collection set (Code 4R1436, Baxter). Packed RBCs were prepared by centrifugation at 5,000 × *g* for 5 minutes at room temperature, which was followed by the removal of sufficient plasma to achieve a Hct of 75 to 80 percent. ABO-matched RBCs were then pooled in groups of 4 units in 1-L PVC bags (4R2032), mixed thoroughly, and distributed equally by weight into four 600-mL PVC transfer bags (4R2023) by use of the sterile connecting device. AS (300 mL) was then added to the RBCs. After mixing, each unit was kept at room temperature (approx. 22°C) for 1 hour.

Study 2: EAS-61 variant units. Standard units (450 ± 45 mL) of blood were collected from each donor into 63 mL of CPD in the primary PVC bag of a collection set with a WBC-reduction filter included (RS-2000, Code 4R3303, Baxter). After WBC reduction, packed RBCs were prepared by centrifugation at 5000 × *g* for 5 minutes at room temperature, which was followed by the removal of sufficient plasma to achieve a Hct of 75 to 80 percent. ABO-matched RBCs were then pooled in groups of 4 units in 1-L sterile bags (4R2032), mixed thoroughly, and distributed equally by weight as described above. EAS (200 mL) was then added to the packed RBC aliquots. The units were mixed and kept at room temperature as in Study 1.

Study 3: AS-3 and EAS-61 units. Standard units (450 ± 45 mL) of blood were collected from each donor into 63 mL of CP2D in the primary PVC bag of an AS-3 double-bag collection set (Code 762-54, Pall Corp., Covina, CA). Packed cells were prepared by centrifugation at 5000 × *g* for 5 minutes at room temperature, which was followed by the removal of sufficient plasma to achieve a Hct of 75 to 80 percent. Four units of identical ABO type were then pooled and realiquoted as described above, and either 100 or 200 mL of AS-3 or 100 or 200 mL of EAS-61 was added.

All units were gently mixed by inversion approximately 30 times, sampled aseptically for in vitro testing, and placed upright in refrigerated storage (1-6°C) 4 hours or less after processing.

In vitro measurements

After gentle mixing by inversion, samples from stored units were collected by a sterile sampling procedure in a biologic safety cabinet. A battery of in vitro tests was performed on all units at the beginning of storage and weekly thereafter.

For Studies 1 and 2, performed at the Hoxworth Blood Center, the total Hb concentration, WBC counts, and RBC counts were measured with a clinical hematology analyzer (MaxM Coulter Counter, Coulter Electronics, Hialeah, FL). Supernatant Hb was measured by using 3,3′,5,5′-tetramethylbenzidine (Procedure No. 527, Sigma Diagnostics, St. Louis, MO). The average degree of RBC shape change from discocytes to echinocytes to spherocytes was measured as RBC morphology scores on 200 cells, determined by a modification of the method of Usry et al.[14] The percentage of hemolysis was determined by the ratio of free Hb to total Hb. The results are expressed as a percentage of hemolysis to compensate for the differences in storage Hct and Hb concentrations in samples. Centrifuged microhematocrits (Clay Adams, Becton Dickinson, Rutherford, NJ) were performed to prevent osmotic changes in cell volume caused by the isotonic diluent used in the blood analyzer. MCV was calculated from the centrifuged microhematocrit and the RBC count. Microvesicle protein was measured as previously described.[15]

RBC ATP concentrations and whole-blood lactate concentrations were measured in deproteinized supernatants. Whole-blood or packed cell aliquots were mixed with cold 12-percent trichloroacetic acid to precipitate blood proteins and centrifuged at 2700 × *g* for 10 minutes, and the protein-free supernatant was frozen at –80°C until it was tested. ATP was assayed enzymatically with a commercially available test kit (Procedure 366-UV, Sigma).

In Study 1, intracellular and extracellular pH was measured with a benchtop pH meter (Orion 900A, Orion Research, Boston, MA) at 22°C. Blood pH has a temperature coefficient of –0.015 pH units per °C, so pH measured at 22°C will be about 0.22 pH units higher than that measured at 37°C. Blood gases and pH were measured in the second study on a blood gas analyzer (855, Corning, Ithaca, NY). Thus, pH was measured at 37°C.

In Studies 1 and 2, supernatant potassium, glucose, lactate, and inorganic phosphorus testing was performed by standard methods at a licensed central laboratory (Health Alliance Laboratories, Cincinnati, OH).

For Study 3, performed at Walter Reed Army Institute of Research, the total Hb concentration, WBC counts, and RBC counts were measured with a different clinical hematology analyzer (Hematology Cell Counter System Series 9110+, Baker, Allentown, PA), but the centrifuged microhematocrit was used to correct for cell swelling. Supernatant Hb was measured spectrophotometrically by using a modified Drabkin assay.[16]

RBC ATP and lactate concentrations were measured in deproteinized packed cells and whole blood, respectively. Whole-blood or packed cell aliquots were mixed with cold 10-percent trichloroacetic acid to precipitate blood proteins and centrifuged at 2700 × *g* for 10 minutes, and the protein-free supernatant was frozen at –80°C until it was tested. ATP was assayed enzymatically with a commercially available test kit (Procedure 366-UV, Sigma).

Blood gases and pH were measured on a blood gas analyzer (855, Corning). The pH was measured at 37°C. Phosphate, lactate, and glucose were measured on a programmable chemical analyzer (Hitachi 902, Boehringer-Mannheim Corp., Indianapolis, IN).

Statistical analysis

Comparisons of means of measured values at given times within the trials were evaluated with the ANOVA using procedures that take account of the weekly repeated measures. Probabilities less likely than 0.05 were considered significant.

RESULTS

In Study 1, the replacement of sodium chloride by sodium bicarbonate was associated, in a linear and dose-dependent manner, with increased RBC ATP concentrations at the end of storage (Fig. 1A). The increases in RBC ATP concentrations were temporally associated with increased glucose consumption (data not shown) and increased lactate production (Fig. 1B). This increased rate of glycolysis occurred in cells stored in solutions with a higher extracellular pH (Fig. 1C), which in turn resulted in a higher intracellular pH (Fig. 1D). The concentration of bicarbonate in the supernatant was increased in proportion to the amount added, and it declined linearly with the length of storage (Fig. 1E). As bicarbonate was broken down, CO_2 was released, and the PCO_2 in the bag initially increased and then declined as the CO_2 diffused from the bags (Fig. 1F). There was no significant differences in RBC morphology or hemolysis with storage in the different solutions (Figs. 1G and 1H). Hemolysis at 10 and 11 weeks was over 2 percent in this study, in which RBCs were stored without WBC reduction.

In Study 2, successive increases in sodium chloride concentration were associated with decreases in MCV and the RBC morphologic index and increases in the shed microvesicle protein in the storage supernatant (Table 2). The decrease in RBC ATP concentration was statistically significant only for the highest dose (Table 2 and Fig. 2A). This change in RBC ATP concentration was not associated with any change in extracellular pH (Fig. 2B) or lactate production (Fig. 2C). At the highest sodium concentration, there was increased hemolysis in the last 2 weeks of storage (Fig. 2D). WBC-reduced RBCs stored in 200 mL of EAS-61 had 0.4-percent hemolysis at 9 weeks and 0.7-percent hemolysis at 11 weeks.

In Study 3, EAS-61, an alkaline AS, caused RBC ATP concentrations to increase during the first 3 weeks of storage (Fig. 3A). This increase was less with 100 mL of AS-3 and did not occur with 200 mL of AS-3 (Fig. 3A). These increased concentrations of RBC ATP were temporally associated with increased supernatant pH (Fig. 3B) and increased lactate production (Fig. 3C). After the pH of the suspending solutions became equivalent at 4 weeks of storage, the rates of lactate production, indicated by the slopes of the lactate concentration plots in Fig. 3C, are only slightly greater in EAS-61. However, storage in EAS-61 was associated with better RBC morphology, and 200 mL of this additive was better than 100 mL (Fig. 3D). Hemolysis was lower in EAS-61 than in AS-3, and the 200-mL volume of each additive solution was better than the 100-mL volume in this regard (Fig. 3E). RBCs stored for 9 weeks in EAS-61 without WBC reduction had 0.4-percent hemolysis.

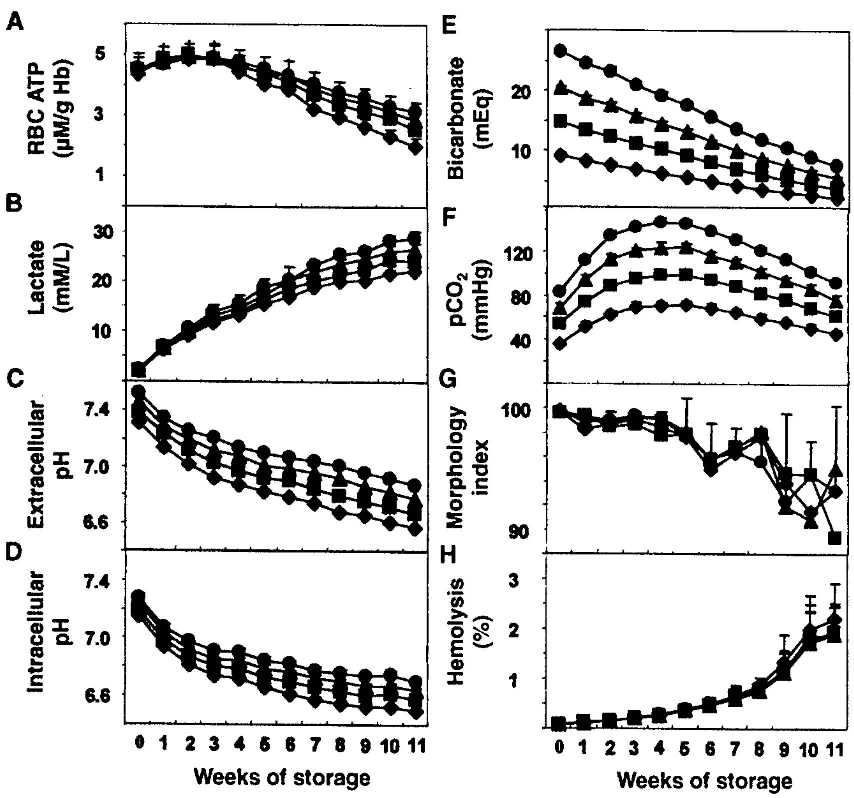

Fig. 1. The effect of increasing concentrations of sodium bicarbonate on A) RBC ATP content, B) whole-blood lactate concentration, C) extracellular pH, D) intracellular pH, E) supernatant bicarbonate concentration, F) supernatant pCO_2, G) RBC morphology index, and H) percentage of hemolysis. The individual solutions contain 0 (◆), 10 (■), 20 (▲), and 30 (●) mEq per L of sodium bicarbonate. Data are presented as mean ± SD; pH was measured at 22°C.

DISCUSSION

We have previously shown that it is possible to make ASs for 9- and 10-week RBC storage.[12,13] These solutions are mixtures of saline, adenine, glucose, mannitol, and disodium phosphate. The solutions are mildly hypotonic to induce cell swelling

TABLE 2. Outcomes of Study 2 after 9-week storage*

	EAS-61 variants with increasing NaCl content			
Measures	EAS-61 (26 mEq)	EAS-71 (50 mEq)	EAS-72 (100 mEq)	EAS-73 (150 mEq)
MCV (fL)	105.5 ± 3.2	100.4 ± 2.1	93.4 ± 2.1	88.5 ± 2.1
Morphology index	81.1 ± 7.8	72.1 ± 9.4	61.2 ± 10.6	53.8 ± 12.3
Vesicle protein (mg/dL RBCs)	8.7 ± 1.7	13.6 ± 1.9	26.7 ± 5.1	40.4 ± 7.8
Hemolysis (%)	0.78 ± 0.11	0.73 ± 0.10	0.84 ± 0.18	1.28 ± 0.33
ATP (% of initial)	53 ± 5	54 ± 5	50 ± 6	45 ± 4

* Values expressed as mean ±SD (N = 6).

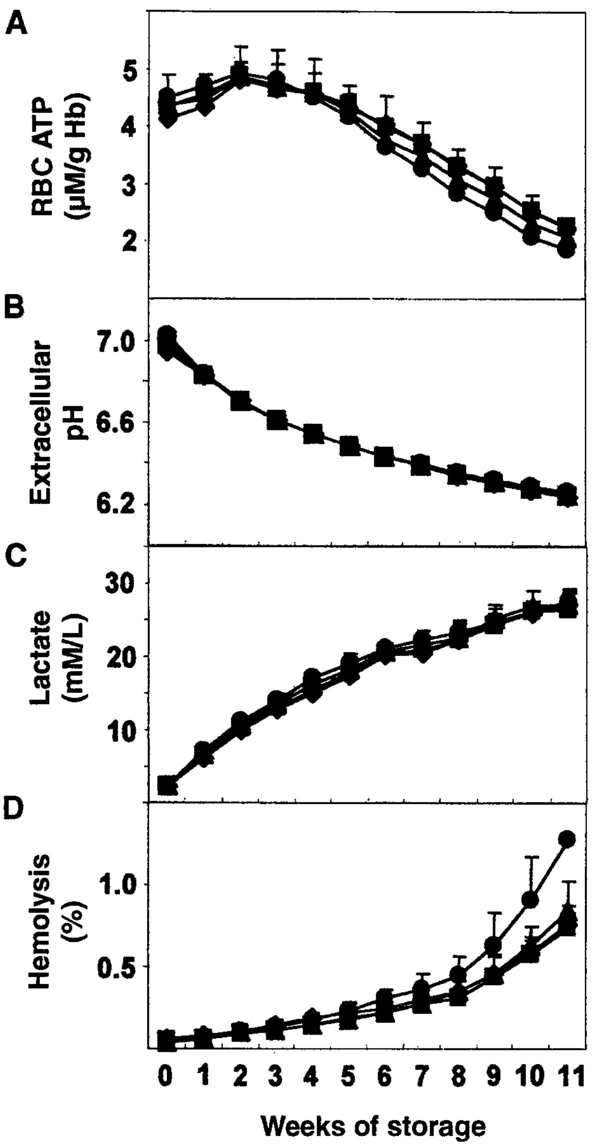

Fig. 2. The effect of increasing concentrations of sodium chloride on A) RBC ATP content, B) extracellular pH, C) whole-blood lactate content, and D) percentage of hemolysis. The individual solutions contain 26 (◆), 50 (■), 100 (▲), and 150 (●) mEq per L of sodium chloride. Data are presented as mean ± SD; pH was measured at 37°C.

and have a pH of about 8.4 as determined by the pK of the disodium phosphate. The solutions appear to work, because they drive RBC ATP synthesis in the first 2 to 3 weeks of storage and because increasing volumes of the ASs are associated with reduced hemolysis. WBC reduction independently reduces hemolysis and is usually necessary when these solutions are used beyond 9 weeks.[17]

To further our understanding and advance the development of these ASs, we have conducted a series of studies to measure the effects of specific changes in solution composition on RBC ATP content and RBC hemolysis. Further, we have tried to improve our understanding of the determinants of altered RBC ATP content by measuring the rates of lactate production and pH decline and of the determinants of hemolysis by measuring changes in RBC morphology and supernatant microvesicle protein.

In Study 1, EAS-64, a 300-mL 10-week AS, was modified by the replacement of sodium chloride with sodium bicarbonate in amounts of 0, 10, 20, and 30 mmol per L, as suggested by the earlier work of Beutler and West.[18] The addition of this salt of a strong base and weak acid makes the solutions more basic initially. The removal of protons in the carbonic anhydrase reaction and subsequent loss of carbon dioxide by diffusion through the plastic bag keep the solutions relatively more basic throughout storage. Lactic acid was produced continuously during storage but at a decreasing rate with time in all the variants of the solution (Fig. 1B). With increasing amounts of bicarbonate, the rate of lactic acid production was better maintained during the course of storage, so that, at the end of storage, the solution with 30 mEq per L of bicarbonate contained 25 percent more lactate. This increased glucolytic activity was associated with increased RBC ATP concentrations after the third week of storage. RBC ATP content is the sum of ATP synthesis and ATP use. The rising ATP concentrations in the first 2 weeks of storage indicate a uniform excess of synthesis in all the solutions. The decreasing concentrations after the third week indicate a net ATP breakdown. If the rate of ATP synthesis is tied to the rate of glycolysis and the rate of breakdown is relatively constant, then the data show how solution pH can be manipulated to maintain RBC energetics, especially at the end of storage. In Study 1, the test units were not WBC reduced, and the hemolysis averaged 2 percent at 10 weeks, as compared to 0.38 percent at 10 weeks in a previous study that included WBC reduction.[13]

In Study 2, EAS-61, a 200-mL 9-week AS, was modified by the addition of sodium chloride so that the resulting solutions had final concentrations of 26, 50, 100, and 150 mmol per L. The addition of sodium chloride had no effect on supernatant pH or the rate of lactate formation, but it did abolish

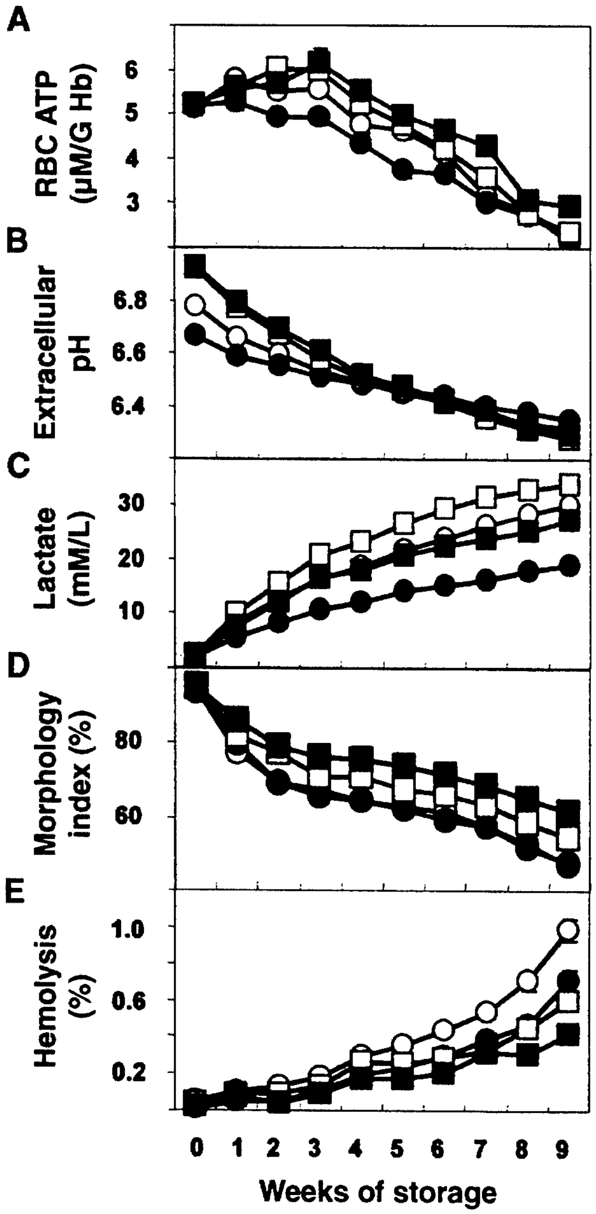

Fig. 3. A comparison of the effects of storage in two different ASs at 2 volumes on A) RBC ATP content, B) extracellular pH, C) whole-blood lactate content, D) morphology index, and E) percentage of hemolysis. The individual solutions and volumes were AS-3 at 100 mL (○), AS-3 at 200 mL (●), EAS-61 at 100 mL (□), and EAS-61 at 200 mL (■). Data are presented as mean ± SEM; pH was measured at 37°C.

cell swelling and led to greater morphologic change, greater loss of membrane microvesicles, and increased overall hemolysis. At the highest salt concentration, the RBC ATP contents were less at the end of storage, which suggests that the shape change and membrane loss were energy-consuming processes.

In Study 3, EAS-61 was compared directly with AS-3 at 100- and 200-mL volumes. EAS-61 has higher pH, but AS-3 has a higher phosphate concentration, which gives it greater buffer capacity. Increasing amounts of AS-3 drove the storage solution pH down, reducing lactate production and preventing the initial rise in RBC ATP concentration. By 4 weeks of storage, the supernatant pH in all the groups was equal and the rate of lactate production became essentially equal. Nevertheless, RBCs stored in the hypotonic EAS-61 had better morphology, and a volume of 200 mL was better than 100 mL in this regard. Both solutions showed reduced hemolysis with increasing storage solution volume, but, at each volume, hemolysis in EAS-61 was approximately half that observed in AS-3.

The EASs, EAS-61 and EAS-64, appear to work to improve storage in several ways. First, they raise the pH in the storage solution and therefore in the intracellular space. The higher intracellular pH appears to drive glycolysis and therefore ATP synthesis in the early weeks of storage, so that the later phase of declining RBC ATP content starts both at a higher maximum and later in the course of storage. Second, relative osmotic hypotonia causes RBC swelling, which in turn improves the morphology and reduces the membrane loss by microvesiculation. The shape change and microvesiculation appear to be energy-consuming processes, and minimizing them appears to conserve ATP for other uses. Finally, as observed previously, increasing the volume of AS reduces the hemolysis in all the solutions we have tested.

The experiments described here suggest several ways to improve these solutions. Replacing some of the sodium chloride with sodium bicarbonate is an obvious first step. Exactly how much bicarbonate to use is less clear, in that more appears to be better from the point of storing the cells, but higher doses may cause clinical problems. Optimizing the tonicity of the solutions relative to the planned AS volume to minimize hemolysis appears to be straightforward also, as the second experiment suggests that optimal tonicity is close to the present formulation of EAS-61 for a 200-mL AS. Finally, other changes in the storage system that increase the initial intracellular pH of the stored cells are expected to be beneficial.[19,20] Obviously, in vitro biochemical measures are only a partial surrogate for measured in vivo recovery and survival of RBCs.[21]

The potentially more difficult question is how best to exploit the success of these solutions. They appear to have the ability to extend to at least 10 weeks and probably beyond the period during which RBCs can be stored with greater than 75-percent recovery and less than 1-percent hemolysis. At the same time, they can reduce the physiologic burden on transfusion recipients due to the consequences of transfusing RBCs with the storage lesion. The exact time of extended storage determines the ratio of these two benefits. The US Army, which paid for this research, would like the additional storage time to improve blood supply logistics. Rational health care economy would suggest that the cost savings would be greater if most of the benefit of these solutions were taken in transfusing cells with less storage damage. Perhaps the lessons learned from these studies can guide further development on a path toward achieving both of these goals.

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Appendix J - (Alkaline CPD and the preservation of red blood cell 2,2-DPG)

Alkaline CPD and the preservation of RBC 2,3-DPG

John R. Hess, Heather R. Hill, Cynthia K. Oliver, Lloyd E. Lippert, and Tibor J. Greenwalt

BACKGROUND: Concentrations of 2,3-DPG decline rapidly in the first week of RBC storage because of the low pH of conventional storage solutions. Alkaline additive solutions, which can preserve RBCs for up to 11 weeks, still do not preserve 2,3-DPG because the starting pH is below 7.2.

STUDY DESIGN AND METHODS: Alkaline CPD (pH = 8.7) was made with trisodium citrate, dextrose, and disodium phosphate. Twelve units of whole blood were collected into heparin and pooled in groups of four units. Each pool was then aliquoted into four units; 63 mL of CPD with pH 5.7, 6.5, 7.5, or 8.7 was added to one unit of each pool, and 300 mL of the alkaline experimental additive solution-76 was added. In Study 2, 12 units were collected into alkaline CPD, pooled in groups of four, aliquoted as described, and stored in four variants of experimental additive solution-76 containing 0, 9, 18, and 27 m*M* of disodium phosphate. RBC ATP and 2,3-DPG concentrations, intracellular and extracellular pH and phosphate concentrations, hemolysis, and other measures of RBC metabolism and function were measured weekly.

RESULTS: RBCs stored in more alkaline conditions made 2,3-DPG, but at the expense of ATP. Concentrations of 2,3-DPG decreased after 2 weeks storage, but ATP concentrations never fully recovered. Providing more phosphate both increased the duration of 2,3-DPG persistence and raised ATP concentrations in the later stages of storage.

CONCLUSIONS: Maintaining both 2,3-DPG and ATP requires both high pH and high concentrations of phosphate.

Better RBC storage solutions can lengthen the duration of storage, increase the recovery of stored cells, and improve RBC function. Lengthening the duration of storage can, in turn, reduce RBC losses from outdating, improve the availability of blood in isolated locations, and increase the utility of autologous blood storage.[1] As improved storage solutions by their nature increase the in vivo recovery of stored cells, they can partially offset RBC losses caused by other changes in transfusion practice, such as the use of WBC-reduction filters.[2] Improved storage solutions can also reduce RBC membrane loss, increase the amount of RBC ATP available for vasoregulatory secretion, and increase the content of 2,3-DPG to improve oxygen delivery.[3-6]

We have developed solutions that allow RBC storage for 9, 10, and 11 weeks.[7-9] These solutions use conventional additive solution ingredients such as saline, adenine, glucose, and mannitol but are made alkaline by the addition of disodium phosphate. Such solutions permit in vivo recovery of 80 percent of cells at the time limits of storage and, presumably, a proportionately greater recovery after shorter periods. They prevent up to

ABBREVIATION: EAS = experimental additive solution.

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Disclaimer: The authors certify that they are employed, directly or indirectly, by the U.S. Army and the University of Cincinnati, which have patent rights on the EAS-76. Two of the authors (JRH and TJG), are listed as inventors on the patent. The opinions expressed herein are the private views of the authors, and are not to be construed as official or as reflecting the views of the U.S. Department of the Army or of the U.S. Department of Defense.

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TRANSFUSION 2002;42:747-752.

80 percent of the RBC membrane loss associated with conventional storage and result in greater RBC ATP concentrations.[8] Both of these effects are expected to improve the microvascular flow characteristics of stored RBCs. However, the new solutions do not improve RBC 2,3-DPG content, which is lost in the first 10 days of storage.

In an attempt to increase further the duration of RBC storage and to maintain the RBC 2,3-DPG content, we alkalinized the CPD. The resulting more alkaline cells maintained supranormal concentrations of 2,3-DPG for 3 weeks, but at the expense of RBC ATP concentrations. This appeared to occur because the total amount of phosphate available inside the RBCs was initially limited. In an attempt to overcome this limitation, more phosphate was added to the experimental additive solution (EAS). With increasing phosphate content, storage solution pH was better maintained. 2,3-DPG was maintained for up to 5 weeks, and ATP concentrations were higher in the later phases of storage. This article describes these two RBC storage experiments in detail and discusses their implications.

MATERIALS AND METHODS

Volunteers

Two groups of 12 healthy volunteers consented to having their RBCs used for storage studies. The donors met standard blood donor criteria and gave informed consent in accordance with protocols approved by the Institutional Review Board of the Walter Reed Army Institute of Research (Silver Spring, MD).

Storage solutions

Alkaline CPD was made with trisodium citrate, 120 m*M;* dextrose, 140 m*M;* and disodium phosphate, 18 m*M* (Table 1). It had a pH of 8.7. Conventional CPD was made from citric acid, 18 m*M;* trisodium citrate, 102 m*M;* dextrose, 140 m*M;* and monosodium phosphate, 18 m*M.* Its pH was 5.7. CPD variants with pH of 6.5 and 7.5 were made by mixing the conventional and alkaline forms.

The EAS-76 solution (composition: NaCl, 45 m*M;* $NaHCO_3$, 30 m*M;* adenine, 2 m*M;* glucose, 50 m*M;* mannitol 30 m*M;* and Na_2HPO_4, 9 m*M*) was prepared in the laboratory from high-purity adenine, sugars, salts, and water that was sterilely filtered into 1-L storage bags (4R2238, Baxter Healthcare, Roundlake, IL). Variants with 0, 18, and 27 m*M* of disodium phosphate were also prepared, and in these variants, the sodium chloride was also adjusted to maintain a constant sodium content (Table 2). The bags were held at 37°C for 2 weeks. At the end of 2 weeks, the solutions were cultured. The cultures were incubated for another 2 weeks. Sterility was confirmed by the absence of bacterial and fungal growth in the cultures after 14 days.

TABLE 1. Composition of CPD variants used in studies (m*M*)*

Composition	Conventional CPD	Alkaline CPD
Citric acid	18	0
Sodium citrate	102	120
NaH_2PO_4	18	0
Na_2HPO_4	0	18
Dextrose	140	140
pH	5.7	8.7

* All solutions used in a 63-mL volume.

Study designs

We conducted two "pooling" studies to evaluate the effect of storage solution ingredients on RBC metabolism and integrity over the course of 12 weeks of storage in PVC bags. Pooling reduces the largest source of variability in conventional blood storage studies, the differences between the RBCs from different donors,[10] by placing some of the cells from each donor in every treatment group of the study while maintaining conventional unit size. RBC units, nonreactive in the IAT, were grouped into sets of four ABO-matched units. Each set was then collected, pooled, mixed, and aliquoted to make four identical pooled units. One unit from each pool went into each of the four arms of the corresponding study.

RBC unit preparation

In Study 1, standard units (450 ± 45 mL) of blood were collected from each donor into 3000 units of heparin in 3 mL of sterile water for injection. Units were WBC reduced (Sepacell, Fenwal, Round Lake, IL), pooled in groups of four, aliquoted, and 63 mL of CPD at pH 5.7, 6.5, 7.5, or 8.7 was added, one variant to one unit from each pool. RBCs were then packed by centrifugation at 5000 × *g* for 5 minutes. The plasma was removed to an Hct of 80 percent, and 300 mL of EAS-76 was added. All fluid additions were performed with a sterile tubing connection device (SCD 312, Terumo, Elkton, MD).

In Study 2, 12 units were drawn into bags containing

TABLE 2. Composition of EAS-76 used in both studies and the variants used in Study 2 (m*M*)

Composition	EAS-76	EAS-76 (0 m*M* PO_4)	EAS-76 (18 m*M* PO_4)	EAS-76 (27 m*M* PO_4)
NaCl	45	63	27	9
$NaHCO_3$	30	30	30	30
Na_2HPO_4	9	0	18	27
Adenine	2	2	2	2
Dextrose	50	50	50	50
Mannitol	30	30	30	30
pH	8.4	8.4	8.4	8.4

* All solutions used in a 300-mL volume.

63 mL of alkaline CPD at pH 8.4. Units were WBC reduced, packed and plasma reduced, pooled, mixed, and aliquoted. Additive solution was then added to the study units, either EAS-76 or a variant with 0, 18, or 27 m*M* of disodium phosphate.

Units were stored upright in a monitored blood bank refrigerator at 1°C to 6°C. Units were sampled weekly for 12 weeks.

In vitro measurements

Samples (15 mL/week) were collected, after gentle mixing by inversion, by a sterile sampling procedure in a biologic safety cabinet. A battery of in vitro tests was performed on all units at the beginning of storage and weekly thereafter.

The total Hb concentration and RBC counts were measured with a clinical hematology analyzer (Hematology Cell Counter System Series 9110+, Baker, Allentown, PA). Supernatant Hb was measured spectrophotometrically with the modified Drabkin assay.[11] Percent hemolysis was determined by the ratio of free to total Hb. The results are expressed as percent hemolysis to compensate for the differences in Hct and Hb concentrations between samples. Centrifuged microhematocrits (Clay Adams, Becton-Dickinson, Rutherford, NJ) were performed to avoid the readjustment of cell volume and subsequent errors caused by the isotonic diluent used in the blood analyzer.

RBC ATP, 2,3-DPG, glucose, phosphate, and lactate concentrations were measured in supernatants of deproteinized RBCs. Cell aliquots were mixed with cold 10-percent trichloroacetic acid to precipitate blood proteins and were centrifuged at 2700 × *g* for 10 minutes, and the protein-free supernatant was frozen at –80°C until tested. ATP and 2,3-DPG were assayed enzymatically with commercially available test kits (Procedures 366-UV and 35-UV, Sigma Diagnostics, St. Louis, MO). Phosphate was measured spectrophotometrically as ammonium phosphomolybdate. RBC intracellular pH was measured on three times-frozen RBC aliquots.

Blood gases and pH were measured on a blood gas analyzer (Corning 855, Ithaca, NY). Thus, pH was measured at 37°C. Extracellular sodium, potassium, chloride, phosphate, lactate, and glucose were measured on a programmable chemical analyzer (Hitachi 902 Analyzer, Boehringer Mannheim, Indianapolis, IN). The average degree of RBC shape change from discocytes to echinocytes to spherocytes was measured according to the method of Usry et al.[12]

Statistical analysis

Comparisons of means of measured values at given times between the arms of the trial were evaluated by ANOVA with adjustments for repeat measures. Probabilities less likely than 0.05 were considered statistically significant.

RESULTS

In Study 1, to prevent collecting whole blood into CPD solutions of increasingly higher pH, whole blood units were collected into heparin, pooled and aliquoted into test units, and the CPD of pH 5.7, 6.5, 7.5, or 8.7 was added. This was followed by a conventional packing of the RBCs, removal of plasma, and addition of 300 mL of the alkaline additive solution EAS-76. The pooling procedure produced sets of units with similar characteristics. However, the CPD solutions of higher pH have low buffer capacity in the physiologic range, and thus, the major observed differences were between conventional CPD of pH 5.7 and each of the more alkaline variants. This is most clearly seen in the RBC ATP concentrations (Fig. 1A), which decreased markedly in the first week of storage in the units containing the more alkaline CPD variants. These more alkaline anticoagulants increased the intracellular and extracellular pH by only 0.1 to 0.2 pH units (Fig. 1B and 1C). The decrease in RBC ATP concentrations was not caused by reduced glycolysis, as evidenced by the slightly higher rates of lactate production in the more alkaline units (Fig. 1D). Rather, the ATP decrease was associated with an increase in 2,3-DPG production (Fig. 1E). Both of these events were associated with a nadir in intracellular phosphate concentration at 7 days of storage (Fig. 1F). Extracellular phosphate concentrations decreased during the first week under all conditions and continued to decrease in the second week with the more alkaline solutions (Fig. 1G). None of these changes had a significant effect on the hemolysis of RBCs during 12 weeks of storage (Fig. 1H) or on the RBC morphology (data not shown).

In Study 2, the effect of adding more phosphate to the additive solution was assessed by using variants of EAS-76 containing 0, 9, 18, and 27 m*M* of disodium phosphate. All units were drawn into alkaline CPD at pH 8.4, WBC reduced, plasma reduced, pooled, and aliquoted, and finally, the additive solution was added. Again, the pooling process gave good statistical resolution between the effects of the different additive solutions. As in Study 1, alkaline CPD caused all of the units in Study 2 to have a decrease in ATP concentration in the first week of storage (Fig. 2A). This initial decrease was greatest in the low phosphate units, and the subsequent rebound was greatest in those units with the highest additive solution phosphate concentration. These higher concentrations of RBC ATP in the higher phosphate units persisted at decreasing levels throughout storage. Intracellular and extracellular pH decreased continuously during 12 weeks of storage (Fig. 2B and 2C). The extracellular pH decreased less in the units with higher additive solution phosphate content despite greater lactate production in those units (Fig. 2D). RBC 2,3-DPG concentrations rose higher and persisted longer in the units stored in the

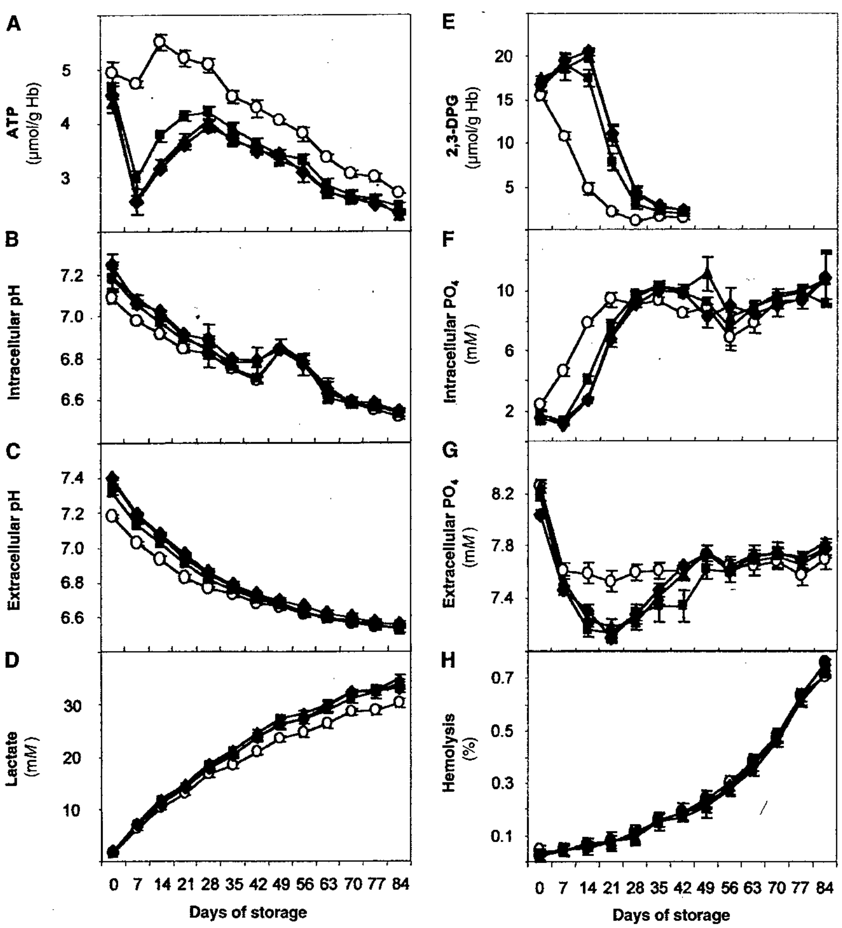

Fig. 1. Weekly measures of (A) RBC ATP concentration, (B) intracellular pH, (C) extracellular pH, (D) RBC lactate concentration, (E) RBC 2,3-DPG concentration, (F) intracellular phosphate concentration, (G) extracellular phosphate concentration, (H) fractional RBC hemolysis for the RBCs collected in CPD at pH 5.7 (-○-), CPD at pH 6.5 (-■-), CPD at pH 7.5 (-▲-), and CPD at pH 8.7 (-♦-). RBC ATP concentrations were reduced at 1 week in the more alkaline CPD variants, whereas the amount of lactate produced was great greater in the alkaline solutions. Data presented as mean ± SEM (n = 3 pooled units/group).

additive solutions with higher phosphate content. The intracellular free phosphate concentrations were very low in all units at the end of the first week but increased thereafter (Fig. 2F). The intracellular phosphate concentrations appeared to come to equilibrium with the extracellular concentrations by approximately 7 weeks of storage (Fig. 2G). Higher additive solution phosphate concentrations were associated with lower hemolysis, but most of the benefit was seen at the 9 m*M* concentration (Fig. 2H).

Electrolyte and blood gas measures had physiologically predictable results (data not shown). Extracellular sodium concentrations were not different at any time point in either study. Extracellular potassium concentrations were lower and increased more slowly in units with higher RBC ATP concentrations. Lower pH led to higher pCO_2 and more rapid decreases in extracellular fluid bicarbonate.

DISCUSSION

There are many reasons to try to improve RBC storage. First longer storage per se can reduce blood bank losses from outdating, improve the usefulness of autologous RBC storage in many clinical situations, improve the availability of RBCs in remote locations, and help ease seasonal shortages of RBCs. Second, as longer storage implies better storage, most transfusion recipients will face a reduced burden of nonviable cells, and many will benefit from cells with better flow properties because of reduced membrane loss and higher RBC ATP concentrations. Third, conventional RBC storage leads to the production and coadministration of membrane breakdown products, such as RBC microvesicles that are procoagulant, and lysophospholipids, such as platelet activating factor, that are proinflammatory. The best way to reduce this exposure is to prevent the formation of the toxic breakdown products in the first place. Finally, as other efforts to improve blood safety, such as filtration WBC reduction, lead to RBC losses in production, these losses can be partially offset by giving cells with better in vivo recovery.

To address these needs, we have developed a family of RBC additive solutions that allow 9-, 10-, and 11-week storage. Prolonged storage was achieved by mapping the response of RBCs to increases in storage solution volume, pH, and concentrations of salt, mannitol, phosphate, and bicarbonate, and exploiting the findings in successive changes in the solutions. The findings were validated at each step by the repetition of successful arms of in vitro studies with human trials of in vivo 24-hour RBC recovery.

Maintaining pH is the most important effect of these solutions. Normally, fresh venous blood has a pH of 7.35 at 37°C (as blood is cooled, the pH increases 0.0147 pH units/°C, or one-half pH unit for cooling from 37°C to 4°C).[13] Conventional anticoagulants, such as CPD at pH 5.7, and additive solutions, such as AS-1 at pH 5.8, reduce the pH of stored blood to approximately 7.0, depressing ATP synthesis from levels seen at higher pH and reducing RBC survival. Our alkaline additive solutions, such as

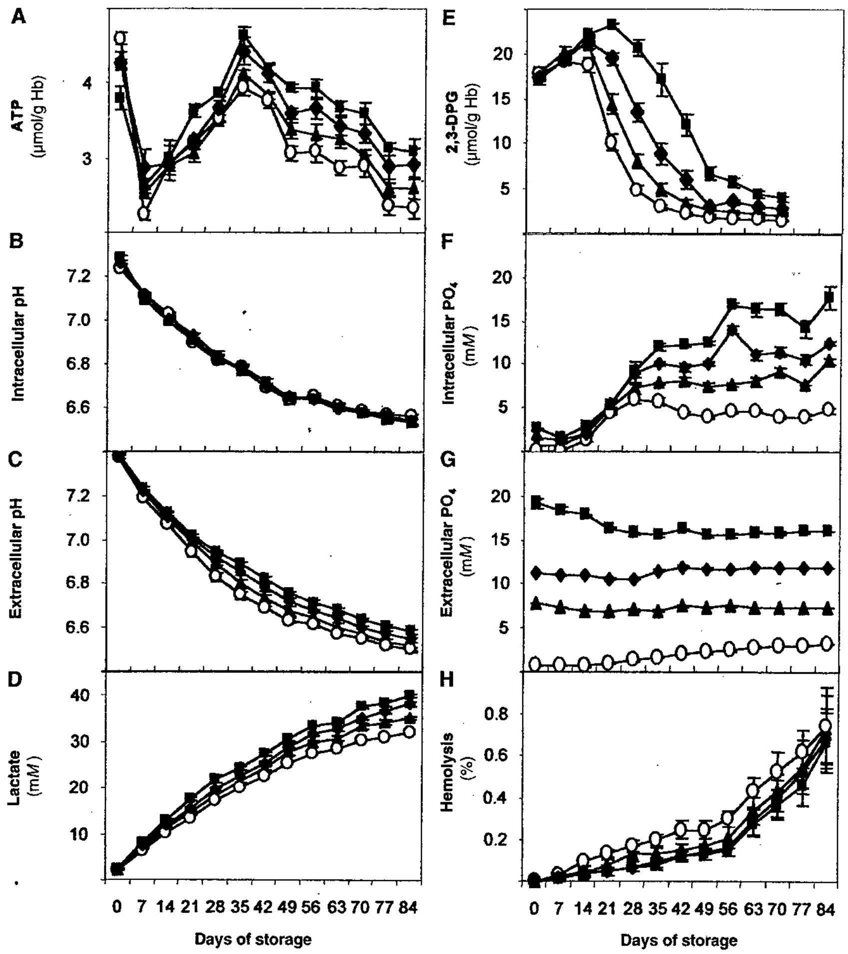

Fig. 2. Weekly measures of (A) RBC ATP concentration, (B) intracellular pH, (C) extracellular pH, (D) RBC lactate concentration, (E) RBC 2,3-DPG concentration, (F) intracellular phosphate concentration, (G) extracellular phosphate concentration, (H) fractional RBC hemolysis for the RBCs collected in EAS-76 variant with 0 m*M* phosphate (-○-), EAS-76 with 9 m*M* phosphate (-▲-), EAS-76 variant with 18 m*M* phosphate (-◆-), and EAS-76 variant with 27 m*M* phosphate (-■-). RBC ATP concentrations were reduced at 1 week under all storage conditions, but recovered to greater concentrations by 5 weeks in the higher phosphate variants. Data presented as mean ± SEM (n = 3 pooled units/group).

EAS-76 with a pH of 8.4, result in an extracellular pH of approximately 7.2 at the beginning of storage. The low, 9 mEq/L phosphate concentration in EAS-76 cannot overcome the high acid load of conventional CPD despite the almost five times greater volume.

In Study 1, we tried to increase further the duration of RBC storage by increasing initial storage pH. As EAS-76 is already at pH 8.4, we chose to raise the pH of the CPD solution from the standard 5.7 to 8.7 by replacing the citric acid with trisodium citrate and the monosodium phosphate with disodium phosphate. We found that collecting the RBC into alkaline CPD did raise the initial pH of the final stored mixture to approximately 7.4. This increase in initial pH was sufficient to increase the production of 2,3-DPG, whose synthesis removes substrate from one of the two ATP-producing steps in glycolysis, phosphoglycerate kinase. When this happens, there is no net ATP production from glucose breakdown. Moreover, the amount of phosphate available in the RBC is limited, and its use in 2,3-DPG production meant that phosphate was not available for ATP production. Under these circumstances, RBC ATP concentrations diminished during the first week of storage and recovered somewhat during the next 3 weeks but never reached the concentrations observed in the units collected in conventional acidic CPD. The lower ATP concentrations occurred despite higher rates of glycolysis, as measured by greater lactate production. The lower RBC ATP concentrations did not cause differences in the rate of hemolysis or the RBC morphology.

In Study 2, the effect of increasing phosphate concentration in the additive solution (0, 9, 18, and 27 m*M*) was assessed in conjunction with collection into the most alkaline CPD variant (pH 8.7). Again, the combination led to a starting pH of approximately 7.4, a period of initial 2,3-DPG synthesis, and a decreased concentration of ATP at 1 week. Over the subsequent weeks, the additive solution variants with higher phosphate concentrations produced better buffering with higher extracellular pH despite greater lactate production at each time point. Inside the RBCs, the pH was not measurably different, but higher phosphate concentrations supported greater and longer 2,3-DPG synthesis and a greater recovery of ATP concentrations after the initial decline. At the highest additive solution phosphate concentration, 2,3-DPG persisted for more than 5 weeks, and the rebound in ATP concentrations was to higher than initial values by Week 5 and persisted above 3 μmol per g of Hb for the remainder of the 12-week storage period. Also, the higher phosphate concentrations were associated with lower hemolysis.

These results are consistent with storage studies of de Verdier et al.[14] and the biochemical measurements of Rose[15] who showed, more than 30 years ago, that 2,3-DPG is synthesized in RBCs when the pH is greater than 7.2. It repeats the work of Beutler et al.[16-18] that lead to the development of the additive solution containing bicarbonate, adenine, glucose, phosphate, and mannitol, but with higher solution volumes that provide additional dilution and buffering. It extends the RBC storage work of Högman et al.,[6] who showed that reducing the amount of acidic CPD and using a neutral additive solution would allow some 2,3-DPG maintenance and 7-week storage. It supports Beutler's contention that increased additive solution phosphate concentrations increases the metabolic rate, although not greatly, and possibly provides a way around his observation that the high metabolic rate is "self-defeating."[18] As Beutler noted, the most important question is whether RBC viability is improved, or at least not made worse, by the early decrease in ATP concentrations.

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Appendix K - (Effect of 23 hours storage at 25°C on the *in vitor* storage characteristics of CPDA-1 packed red blood cells)

Effect of 24-hour storage at 25°C on the in vitro storage characteristics of CPDA-1 packed red cells

J.P. Ruddell, L.E. Lippert, J.G. Babcock, and J.R. Hess

BACKGROUND: Packed red cells (RBCs) warmed above 10°C are generally discarded. Few data exist on the degree of accelerated metabolism and increased hemolysis of packed RBCs allowed to warm.

STUDY DESIGN AND METHODS: Twenty-four CPDA-1 packed RBC units were combined in 3-unit pools and subdivided into 2 test units and a control unit. One test unit from each pool was warmed to 25°C for 24 hours on Day 6 and the other test unit was warmed on Day 20; control units were maintained at 1 to 6°C. RBC and supernatant chemistries and RBC morphology were measured weekly (Days 0, 7, 14, 21, and 28) and on the day before warming (Days 6 and 20).

RESULTS: Warming CPDA-1 packed RBCs accelerated the catabolism of glucose 10-fold and produced concentrations of glucose, lactate, and ATP after 25 days of storage that were equivalent to those in unwarmed units at 35 days. Supernatant sodium and potassium concentrations were corrected partially with warming. RBC morphology transiently normalized with warming and without increased hemolysis; no bacteria growth was detected.

CONCLUSION: One day of 25°C storage of CPDA-1 packed RBCs accelerates essential metabolite breakdown equivalent to 10 days of storage at 1 to 6°C. It does not appear to matter whether the packed RBCs are warmed on Day 6 or Day 20. This information may be useful in determining the acceptability of blood allowed to warm above 10°C.

The American Association of Blood Banks (AABB) and the Food and Drug Administration have established guidelines for the storage and preservation of packed red cells (RBCs). According to AABB Standards G1.300 and G2.000,[1] packed RBCs must be stored at temperatures between 1 and 6°C and shipped at temperatures between 1 and 10°C.[1] The Food and Drug Administration requires the same standards. The Code of Federal Regulations stipulates that blood may not be issued unless it has been stored at 1 to 6°C or maintained at 1 to 10°C during shipping.[2] It further stipulates that blood should be placed in storage at 1 to 6°C immediately after the separation of platelets.[3] Common practice is for RBCs stored at temperatures outside those ranges to be destroyed, though the AABB *Technical Manual* states, "Blood exposed to temperatures above 10°C is not necessarily unsuitable for transfusion."[4(p155)] There are few or no data that support either the destruction of such blood or its retention for transfusion, but the *Technical Manual* does mention an increased possibility of bacterial contamination.[5] This investigation provides data on the accelerated rate of depletion of metabolic support when RBCs are stored near ambient temperature (25°C) for a prolonged period (24 hours) either early (Day 6) or late (Day 20) in the shelf-life of a CPDA-1 packed RBC unit.

ABBREVIATIONS: AABB = American Association of Blood Banks, Hb = hemoglobin; RBC(s) = red cell(s).

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MATERIALS AND METHODS

Donor screening

Twenty-four volunteers, 10 women and 14 men who met all AABB and FDA blood donor criteria and whose blood was further screened and found not to have hemoglobin S (HbS), alloantibodies, or abnormal osmotic fragility, donated a full unit of blood for this study. All volunteers gave their informed consent in accordance with a protocol approved by the Institutional Review Board at Walter Reed Army Institute of Research.

Packed RBCs

Blood (450 mL) was collected into a CPDA-1 double-bag system (4R6210, Fenwal Division, Baxter Healthcare Corp., Deerfield, IL) and held at ambient room temperature before processing. Packed RBCs were prepared within 2 hours of whole-blood collection by centrifugation at 5000 × *g* for 5 minutes at room temperature and removal of plasma to achieve an average hematocrit of 67 percent (range, 66-68%). Three units of packed cells of the same ABO group were pooled by serial sterile connections to a 1.0-L transfer bag (4R6210, Fenwal). The contents were mixed and divided by weight into three aliquots in 400-mL satellite bags (PL-146) from a CPDA-1 quadruple-bag collection system (4R6423P, Fenwal) connected by use of a sterile connecting device (SCD312 Sterile Tubing Welder, Terumo Medical Corp., Elkton, MD) to the pooling bag. The pooled packed cells were mixed by gentle inversion of the transfer bag along its long axis at least 15 times. All transfers to or from the pooling bag were made by gravity. The three aliquots from each pool—the Day 6 early-warmed test unit, the Day 20 late-warmed test unit, and the control unit—were placed in refrigerated storage (1-6°C) within approximately 4 hours of collection and stored continuously at 1 to 6°C in a monitored refrigerator, except during the planned warming periods.

Sampling

Samples were taken at the time of pooling (Day 0) from each of the eight pools; all other samples were taken from the 24 individual units. A sampling site coupler (4C2405, Fenwal) was used to collect samples from the pools on Day 0 and from the test and control units on Day 35. An alternative method was used to collect the intervening samples on Days 6, 7, 14, 20, 21, and 28. Briefly, a length of integrally attached tubing was filled with mixed blood and clamped approximately 2 inches from the distal end. The distal end was cut with scissors and the clamp was released while light pressure on the blood bag was maintained. The required amount of sample was expelled, and the tubing was reclamped and heat-sealed proximal to the clamp. The tubing was then restripped and reclamped. All sampling was performed in a biological safety cabinet (Model #NU 408FM-400, Nuaire, Plymouth, MN).

Incubation of test units

On Day 6, all test and control units from each pool were sampled; test units labeled for early warming were removed from refrigerated storage and placed in a 25°C incubator for 24 hours. On Day 7, at the end of the incubation, all test and control units were sampled and returned to refrigerated storage. On Day 20, all test and control units were sampled again; this sampling was followed by warming (25°C, 24 hours) of those units designated late-warmed test units. On Day 21, at the end of the incubation, all units were again sampled and returned to refrigerated storage.

Testing

The pH of the packed RBCs was measured with a clinical blood gas analyzer (855 Blood Gas Analyzer, CIBA-Corning, Medfield, MA). The Hb and Hct were measured with a clinical hematology analyzer (Cell Counter System Series 9000, Baker Hematology, Allentown, PA); plasma sodium and potassium were determined by using ion-specific electrodes in a clinical chemistry analyzer (Cobas Fara, Roche Diagnostic Systems, Nutley, NJ), all in accordance with manufacturer's directions. Whole-blood glucose, total ATP, and lactate levels were obtained from deproteinized samples. Briefly, proteins in whole-blood samples were precipitated with 12-percent perchloric acid on ice, and the supernatant was harvested after centrifugation at 2700 × *g* for 10 minutes. The portion of the supernatant used for glucose and ATP testing was adjusted to pH 8 to 9 with solid $KHCO_3$. The remainder of the deproteinized sample was used for lactate testing and the pH was not adjusted. Glucose levels were determined spectrophotometrically in the Cobas Fars. Total ATP was assayed enzymatically with a commercially available test kit (Kit # 35-B, Sigma Diagnostics, St. Louis MO). ATP levels were reported as µmol per g of Hb. The lactate levels were measured with a commercially available quantitative lactate kit (#228, Sigma Diagnostics) adapted to a clinical chemistry analyzer (Roche).

The RBC morphology score was determined according to the method of Usry et al.[6] Briefly, the RBCs were fixed with gluteraldehyde, examined microscopically, and categorized into one of six morphologic types, ranging from discocyte to spherocyte. After at least 200 cells were categorized, the number of each morphologic type was multiplied by a weighting factor, ranging from 1.0 for the discocyte to 0.0 for the spherocyte; the summed weighted score was divided by the number of cells scored and reported as a percentage.

The plasma or supernatant Hb was measured spectrophotometrically at 540 nm by the method of Moore et al.,[7] which adapts the classical cyanmethemoglobin method to the lower levels of Hb found in the plasma or supernatant of stored blood. The supernatant (plasma) Hb level was divided by the total Hb level and multiplied by 100 to determine the percentage of hemolysis, by the formula:

% Hemolysis = (supernatant Hb in g/dL/total Hb in g/dL) × 100.

Sterility

The sterility of all aliquots was assessed in two ways. The first was by visual comparison of the contents of a filled tubing segment prepared soon after collection and the contents of the bag, for any color changes or appearance of bubbles on all sampling days. In the second method, a commercially available blood culture system (Septi-Chek Blood Culture Bottle and Slide, Becton Dickinson Microbiology Systems, Cockeysville, MD) was used on Day 35 of storage to determine if bacteria growth was present.

Data analysis

One-way ANOVA was used to analyze the data. Two comparisons were made. First, the Day 35 means of all values for all three groups were compared. In the second comparison, the Day 28 means of the test groups were compared to the Day 35 mean of the control group for each value. Comparisons with probabilities less than 0.05 were considered significant.

RESULTS

Under all conditions of storage, the extracellular glucose concentration decreased with time. During 35 days of storage at 1 to 6°C, the mean glucose concentration declined from 391 ± 4 to 72 ± 13 mg per dL, averaging a decline of about 9.1 mg per dL per day (Fig. 1A). The glucose concentration declined at the much greater mean rates of 90 mg per dL per day on the days of 25°C storage, 111 ± 5 mg per dL on Day 6, and 70 ± 2 mg per dL on Day 20. This caused the glucose concentrations to be significantly lower in the warm-stored test units at all time points after storage. Increases in plasma lactate and decreases in pH followed the changes in glucose concentration (Fig. 1B and 1C).

The ATP concentrations showed a more complex pattern, initially increasing during the first week of storage and decreasing thereafter (Fig. 1D). The rates of decrease in ATP concentration were observed to be greater in the week after warming than in the continuously refrigerated control. Thus, the unwarmed control units had a significantly higher mean ATP concentration on Day 35 (2.4 ± 0.1 µmol/g Hb) than the warmed test units had on either Day 28 (1.9 ± 0.1 µmol/g Hb, p= 0.012) or Day 35 (1.4 ± 0.1 µmol/g Hb, $p < 10^{-7}$). One warm-stored test unit had an ATP concentration of 1.4 µmol per g of Hb on Day 28; all others were 1.7 µmol per g of Hb or greater.

Mean supernatant plasma Hb concentrations appeared to increase after warming, especially in the units warmed on Day 20, but the differences between the warmed (test) and control units never achieved significance (Fig. 1E). Hemolysis never exceeded 1 percent. Small increases in extracellular sodium and decreases in extracellular potassium that occurred during the warm-storage days persisted through most of storage (Figs. 1F and 1G).

Warming led to significant improvement in RBC morphology scores during the 24 hours of warming, but the improvement was not maintained in subsequent weeks (Fig. 1H). On Day 35, the morphology indexes of the three groups were not different. A comparison of the relative numbers of the morphologic forms of RBCs seen before and after warming on Day 20 showed the reappearance of greater numbers of less-deformed RBCs and disappearance of the more spicular echinocytes. There was no corresponding increase in supernatant Hb to suggest lysis.

Visual examination of all pools and all test and control units revealed no abnormalities that might have indicated bacterial contamination. Broth cultures of all units begun on Day 35 and examined for 4 days thereafter showed no bacteria growth.

DISCUSSION

Liquid storage systems for packed RBCs are licensed for their ability to preserve cells at 1 to 6°C. Efforts to ensure the quality of these RBC components have led to standards and regulations stipulating that blood be stored in continuously monitored refrigerators at 1 to 6°C and transported on wet ice so that its temperature would not rise above 10°C.[1,2] The rule of thumb that has evolved maintains that blood which is outside of a refrigerator for more than 30 minutes should be discarded, because the temperature will have risen above 10°C. The net effect of these rules is to ensure that each unit of blood has been handled in a highly standardized way that has been demonstrated to meet accepted criteria for viability and circulation of posttransfusion RBCs.

Rigid enforcement of these standards, in the face of the knowledge that "exposure to temperatures above 10°C does not necessarily render blood unsuitable for transfusion,"[4(p155)] may lead to several undesirable effects. First, units of blood are regularly discarded for potentially insignificant deviations from the standards. This has led to the proliferation of devices to measure the temperature of blood units that are outside of the refrigerator for short periods. Second, the reluctance to remove blood from monitored refrigeration because of concerns about potential violation of the standards sometimes compromises the availability of blood at scenes of emergency care, even in major hospitals. Third, in military and civil emergency situations, no information is available to the medical directors of blood services about the safety implications for recipients of blood stored in current storage solutions under nonstandard conditions.

Refrigeration preserves RBCs because glycolysis and other metabolic processes are slower at lower temperatures. At 37°C, RBCs consume glucose at a rate of 0.014 to 0.028

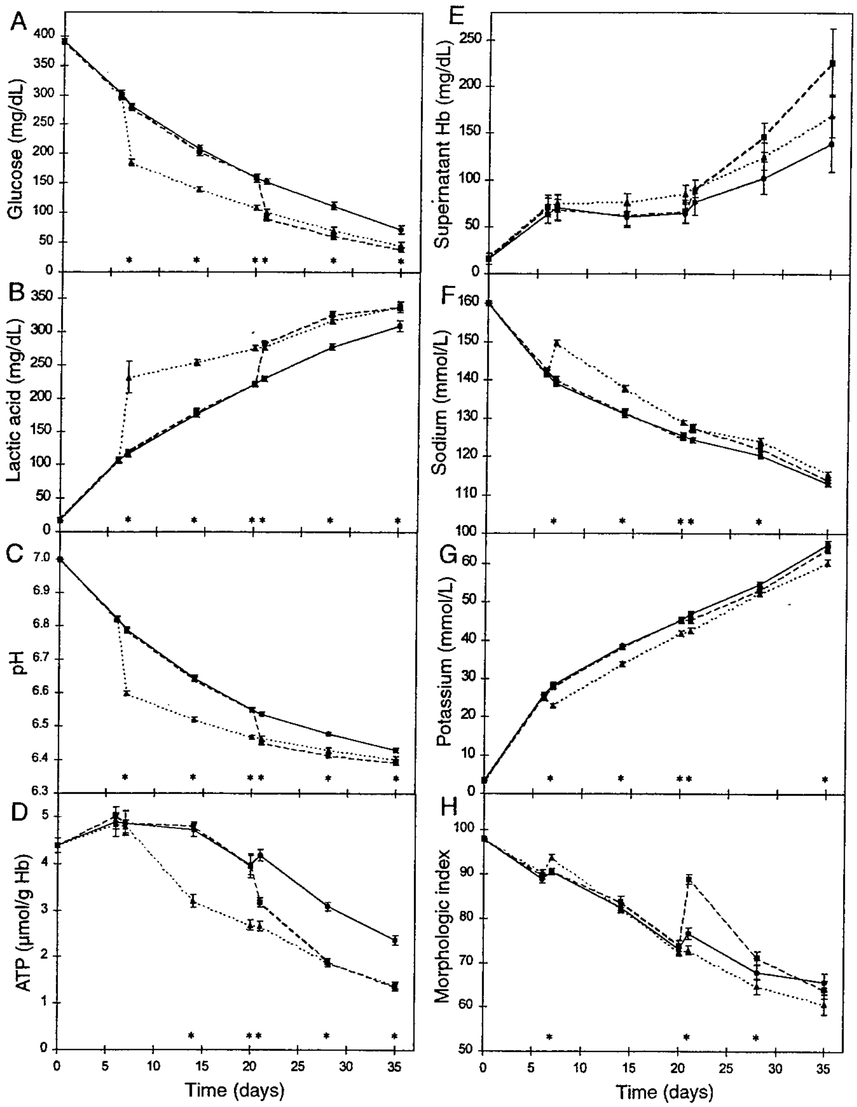

Fig. 1. Concentrations of metabolites, metabolic by-products, supernatant Hb, supernatant electrolytes, and the morphologic index of CPDA-1 packed RBCs either warmed at 25°C for 24 hours at Day 6 (test) (···▲···) or Day 20 (--■--) or stored continuously at 1 to 6°C (control) (—●—). *, the time points where differences in group means could not be due to chance alone at a probability of p = 0.05. Error bars depict ± SEM.

We performed this work to quantitate the acceleration in consumption of glucose and the resulting changes in cellular ATP concentration that occur with increased storage temperature. Our inspiration was the work of Shields,[9] who in 1970 showed that, for whole blood stored in ACD, a day at 22 to 25°C reduced RBC recovery measured with ^{51}Cr by an amount equivalent to the recovery after about a week of 1 to 6°C storage. Because of the large interdonor variation in blood storage characteristics, Shields was able to demonstrate significant differences in recovery only at times beyond the conventional expiration of the units at 21 days. In our study, measuring the metabolite concentrations in aliquots of pooled units allowed the accurate assessment of differences resulting from changes in storage conditions. However, pooling precludes autologous RBC recovery and survival measurements, and estimates of the clinical significance of this work depend on the implications that can be drawn from the cellular ATP concentrations and morphologic measures.[10]

The relationship of cellular ATP concentrations to a 24-hour RBC recovery of 75 percent is probably better understood for CPDA-1 than for any other storage solution. First, in the CPDA-1 licensing study,[11] the mean ATP concentration was 1.93 μmol per g of Hb. Second, Beutler and West[12] have shown that removing glucose from CPDA-1 units by removing plasma reduces 24-hour posttransfusion survival. Third, adding more glucose and adenine to CPDA-1, as is done with the preservative solution CPDA-2,[13] produces a solution that allows RBCs to be stored for a week longer, with equivalent recovery and survival. Thus, the survival of RBCs stored in CPDA-1 is glucose- and ATP-limited, and is most highly correlated with maintaining ATP concentrations above a value of about 2 μmol per g of Hb.[11]

μg per hour per 10^6 RBCs,[8] which is equivalent to a decrease of 7 to 14 mg per dL per hour in the extracellular glucose concentration of packed RBCs stored in CPDA-1 at a hematocrit of 67 percent. Thus packed RBCs stored at body temperature in CPDA-1 would be expected to consume all of the available glucose in 1 to 3 days. Cooling to 1 to 6°C reduces the rate at which extracellular glucose concentration is consumed to the observed 9 mg per dL per day and allows storage for 35 days. Intermediate temperatures should be associated with intermediate rates of glucose consumption and intermediate periods of effective storage.

The morphologic changes in the RBCs that we observed with warming and recooling were striking. During storage in the cold, RBCs slowly evolve from biconcave discs to sharply spicular echinocytes. These changes revert with warming, but after recooling, their reappearance is accelerated. Thus, the cellular injury that underlies the morphologic changes has both reversible and irreversible aspects. Electron micrographs of microvesicles budding from the tips of echinocytic spicules[14] suggest a relationship between RBC shape change and the loss of RBC membrane area that is critical for survival. Warming may provide a model for studying the mechanism of these events.

We have described the effects of a specific time and temperature stress on the performance of the CPDA-1 storage system. Interpolation can serve as a basis for estimates of the effects of stresses of shorter duration or lower temperatures. Thus, if the catabolism of glucose and ATP is a linear function of the time of exposure to the temperature stress, then a single 2-hour exposure to 25°C might be expected to reduce the storage life of a unit of CPDA-1 RBCs by a day. The study also provides a model for measuring the robustness of storage systems and for determining the circumstances under which a measurable effect will be achieved. Small variances from the current standards of practice, such as exposure to temperature of less than 25°C for periods of less than 2 hours, are unlikely to have measurable influence on the quality of the component. If medical directors are called upon to provide blood in emergencies, the study provides some information to guide them in deciding the extent of attempts to take advantage of the performance characteristics of the storage system in their efforts to maximize the availability of blood to save lives.

The authors are not advocating the abandonment of current standards for RBC storage. Those standards provide excellent assurance of the physiologic quality of stored cells, as well as protection against the uncommon event of bacterial contamination. While we saw no evidence of bacterial contamination with broth culturing of all 24 units, even in the face of repeated sampling that began in the first week of storage, data from a small number of units collected in a research setting cannot be applied to clinical blood bank practice.[15,16] Any attempt to take advantage of the performance characteristics of current storage systems through nonstandard usage must be accompanied by heightened vigilance.

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Appendix L - (The viability of autologous human red cells stored in Additive Solution-5 and exposed to 25°C for 24 hours)

The viability of autologous human red cells stored in additive solution 5 and exposed to 25°C for 24 hours

T.J. Reid, J.G. Babcock, C.P. Derse-Anthony, H.R. Hill, L.E. Lippert, and J.R. Hess

BACKGROUND: No data exist on the viability of red cells (RBCs) stored in modern additive solution systems and allowed to warm above 10°C.

STUDY DESIGN AND METHODS: In a randomized crossover study, 3 units of blood were collected at least 8 weeks apart from 11 volunteer donors and stored in additive solution 5 (AS-5). Of 3 units from each volunteer, 1 was stored for 6 weeks at 4°C, 1 for 5 weeks at 4°C except for 24 hours at 25°C on Day 14, and 1 for 5 weeks at 4°C except for 24 hours at 25°C on Day 28. Units were sampled periodically during storage; at the end of storage, viability was measured by the $^{99m}Tc/^{51}Cr$ double-label method.

RESULTS: RBC viability was not significantly different in the storage protocols. Less than 1 percent of stored cells hemolyzed. RBC ATP concentrations at the end of storage correlated with viability and were approximately equal in the warmed units after 30 days' storage and the conventionally stored units after 42 days.

CONCLUSIONS: The data suggest that RBCs stored in AS-5 and allowed to warm to 25°C for 24 hours lose about 12 days of their shelf life.

Red cells (RBCs) are normally stored in the refrigerator and shipped on wet ice.[1] This practice maintains the viability of transfused RBCs and limits bacterial growth. It has been codified in Food and Drug Administration (FDA) regulations,[2] which state that RBCs may not be issued unless they have been stored at temperatures between 1 and 6°C and shipped at temperatures between 1 and 10°C.

Two groups have examined the viability of RBCs stored at room temperature. In 1947, Gibson and colleagues[3] described extensive studies of the effects of warming RBCs during storage. Donors were given radioactive iron, the radioactive cells were transfused to volunteer recipients, and the recovery of cells at 24 hours was measured. In two of these cases, blood was stored for 24 hours at 20 to 25°C. These units of whole blood were drawn into ACD and Alsever's solution, respectively, stored for 46 hours at 4°C, allowed to stand at room temperature (20-25°C) for 24 hours, and returned to storage at 4°C until their transfusion 18 days after being drawn. The recovery fraction was approximately 72 percent in the ACD-stored unit and about

ABBREVIATIONS: AS-5 = additive solution 5; FDA = Food and Drug Administration; Hb = hemoglobin; RBC(s) = red cell(s).

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30 percent with storage in Alsever's solution. The other study is that of Shields in 1970.[4] Shields enrolled 72 volunteers, collected their blood in ACD, and stored it at 4°C except that one-half of the units were warmed to 22°C for the 24 hours preceding their transfusion. One-third of the units were returned at each of 7, 21, and 28 days after drawing, and recovery was measured with the $^{32}P/^{51}Cr$ double-isotope method. Twenty-four hours of room-temperature storage significantly reduced viability, by an amount approximately equal to the loss of viability seen with an additional week of 1 to 6°C storage. Both Gibson and colleagues and Shields concluded that warm storage reduced viability, but Shields suggested that periods of warming up to 24 hours might be tolerated.

There are no data available on the viability of RBCs stored at room temperature in modern additive solutions.[5] Such evidence might support changes in the regulations regarding storage, such as the recent extension of the room-temperature holding time before the separation of platelets.[6,7] Performance-based standards and regulations that allowed additional storage time outside the range of 1 to 6°C are expected to improve the availability of blood in emergency rooms and surgical suites, while reducing the destruction of units issued but not transfused. Such data may also allow medical directors to make evidence-based decisions about the usability of RBCs after nonstandard storage in emergencies.

To test Shields's rule of thumb, that a day at room temperature reduces RBC viability by a week, we compared the viability of RBCs stored in additive solution 5 (AS-5) at 1 to 6°C for 6 weeks to that of RBCs stored for 5 weeks when cells were warmed to 25°C for 24 hours on Day 14 or Day 28. We performed these steps in a randomized crossover study.

MATERIALS AND METHODS

Volunteers

Fifteen healthy volunteers meeting standard blood donor criteria[1] were enrolled after giving informed consent in accordance with a protocol approved by the Institutional Review Board of Walter Reed Army Institute of Research and the U.S. Army's Human Subjects Research Review Board. Volunteers were tested to exclude sickle hemoglobin trait (Sicklescreen, Pacific Hemostasis, Huntersville, NC) and abnormal osmotic fragility (Unopette Test 5830, Becton Dickinson, Rutherford, NJ).

Study design

The study was conducted by using a crossover design. Each volunteer donated 3 units of blood with at least 8 weeks separating the donations. The 3 units were assigned randomly to storage in the following manner: 1 unit was stored for 6 weeks at 4°C (control unit), a second for 5 weeks at 4°C except for 24 hours at 25°C on Day 14, and a third for 5 weeks at 4°C except for 24 hours at 25°C on Day 28. The primary outcome measurement was the viability, measured as the fractional in vivo recovery of the stored RBCs 24 hours after their return to the donor. We also measured postrecovery RBC survival and the changes in supernatant pH, whole-blood glucose concentration, RBC ATP concentration, and supernatant hemoglobin (Hb).

Blood unit preparation

Four hundred fifty mL (± 10%) of blood was collected into CPD in the primary bag of an AS-5 triple-bag system (Optisol, Terumo Medical Corp., Somerset, NJ). Packed cells were prepared by centrifugation at 5000 × *g* for 5 minutes at room temperature, and this was followed by the removal of plasma and the addition of the AS-5 to achieve a target storage hematocrit of approximately 60 percent. All units were sampled for in vitro testing and placed in refrigerated storage (1-6°C) within 2 hours of collection. Test units were stored for 5 weeks and control units for 6 weeks.

Warming

On the scheduled day of warming, test units were removed from the monitored blood bank refrigerator, sampled as described below, and placed in a 25°C incubator for 24 hours. At the end of the warming period, the units were removed, sampled again, and returned to the blood bank refrigerator.

In vitro measurements

Samples from stored units were collected into a small pouch attached to the residual donor needle tubing by using a sterile connecting device (SCD 312, Terumo). The battery of in vitro tests described below was performed on all units on Day 0 after the addition of AS-5; on Days 13, 14, 21, 27, and 28; and at the end of storage (Day 35 for test units and Day 42 for control units).

Blood pH was measured on stored blood supernatant by using a clinical blood gas analyzer (Model 855, CIBA-Corning, Medfield, MA). Total Hb concentration and RBC counts were measured on stored blood with a clinical hematology analyzer (Hematology Cell Counter System Series 9110+, Baker, Allentown, PA). Supernatant Hb was measured spectrophotometrically by using the modified Drabkin assay.[8] The percentage of hemolysis was determined by the ratio of supernatant Hb to total Hb. Centrifuged microhematocrits (Clay Adams, Becton Dickinson, Rutherford, NJ) were performed to ascertain stored-unit volume fractions.

Whole-blood glucose and RBC ATP concentrations were measured in supernatants of deproteinized whole blood. Whole-blood aliquots were mixed with cold, 10-percent trichloroacetic acid to precipitate blood proteins and centrifuged at 2700 × *g* for 10 minutes, and the protein-free supernatant was frozen at –80°C until it was tested. Glucose concentrations were determined with a clinical chemistry analyzer (Cobas Fara, Roche Diagnostic Systems, Nutley, NJ). Glucose consumption was determined from changes in whole-blood glucose concentrations corrected for RBC volume fraction. ATP was assayed enzymatically by using commercially available test kits (Procedure 366-UV, Sigma Diagnostics, St. Louis, MO). In studies in our laboratory, 10- and 12-percent trichloroacetic acid precipitates gave equivalent RBC ATP concentrations (data not shown).

Check for bacterial contamination

Three to 4 days before the end of storage, aliquots from each unit were tested for bacterial contamination in broth and agar cultures by using a commercial blood culture system (Septi-Chek Blood Culture Bottle and Slide, Becton Dickinson Microbiology Systems, Cockeysville, MD). Absence of growth in the cultures was documented before the unit was sampled for return.

In vivo RBC recovery and survival measurement

After 5 or 6 weeks of storage, in vivo RBC recovery was measured 24 hours after autologous return by using both single- and double-radioisotope procedures.[9,10] In brief, a sample of the stored blood was labeled with ^{51}Cr. Concurrently, a fresh blood sample was collected from the volunteer and labeled with ^{99m}Tc. Carefully measured aliquots of the radiolabeled RBCs were mixed and rapidly returned. Blood samples were collected 5, 7.5, 10, 12.5, 15, 20, and 30 minutes after the return and again at 24 hours, 7 days, and 14 days after return. Radioactivity of the samples was measured in a gamma counter (Model 1480, Wallac, Turku, Finland), except for samples from two volunteers, in which the ^{99m}Tc counts were performed in a different counter (CLINGAMMA Model 1272, Wallac). Gamma emissions from ^{99m}Tc-radiolabeled cells were measured in the samples collected during the 30 minutes after return and used to determine the RBC volume at the time of return. The activity from ^{51}Cr-labeled cells was measured on all samples and used to calculate a second RBC volume and the recovery and survival of the returned RBCs. Survival was calculated as the fraction of cells recovered at 24 hours that were still in circulation at 7 and 14 days by using the 1-percent-per-day estimate of ^{51}Cr elution from RBCs.[11]

Statistical analysis

One-way ANOVA was used to examine the relationships between outcomes and mode of storage as well as the differences in measures among the donors. Pearson correlation analysis was used to examine the effect of end-of-storage RBC ATP concentration on these relationships. These calculations were performed with software (Systat 6.1, SPSS, Chicago, IL). Calculations of group means and their standard errors were performed with software (Excel for Windows 97, Microsoft Corp., Redmond, WA). A probability of less than 0.05 was considered significant.

RESULTS

Volunteers

Eleven of 15 enrolled volunteers completed the study. They are described in Table 1. Of the volunteers who withdrew, one was removed from the study because of a persistently low hematocrit after the first donation, one moved from the metropolitan area, and two withdrew for personal reasons.

Uniformity of RBC unit preparation

Storage hematocrit of the AS-5 units varied from 55 to 68 percent, averaging 62 ± 1 percent (mean ± SEM). The mean hematocrits of the stored units were not different among the storage categories or the volunteers.

In vitro measures of RBC metabolism and breakdown during storage

Whole-blood glucose, pH, RBC ATP concentrations, and free Hb were measured at specified times in the course of storage. The whole-blood glucose concentration decreased at an average rate of 7.4 mg per dL per day during storage at 4°C (Fig. 1A). This rate corresponds to 0.028 μM glucose per mL of RBCs per hour. Warming the cells to 25°C increased the rate of decrease of whole-blood glucose concentration 12-fold, to 86 mg per dL per day. The pH also decreased and in a manner similar to that of glucose concentration (Fig. 1B). RBC ATP concentrations decreased

TABLE 1. Characteristics of volunteers who completed the study

Number	Age	Sex	Hematocrit: Donor*	Hematocrit: In units†	Diseases, drugs, or other abnormality
1	31	M	40/42/41	57/65/62	Loratadine/pseudoephedrine
2	44	M	47/47/45	64/63/68	
3	32	M	40/42/41	62/60/60	Propranolol, fluoxetine
4	33	M	43/40/43	62/68/63	Type II diabetes, no treatment
6	30	M	41/40/36	57/62/60	Treated alcoholism
10	27	M	39/40/40	62/62/62	
11	53	M	42/43/41	62/61/58	
12	27	M	41/39/43	57/62/65	
13	41	F	40/40/40	64/65/64	
14	48	M	41/40/41	62/62/66	
15	44	M	48/46/49	58/55/62	

* Hematocrit at donation of the unit to be stored at 4°C for 5 weeks with warming on Day 14/of the unit to be stored at 4°C for 5 weeks with warming on Day 28/of the unit stored at 4°C for 6 weeks (control unit).

† Hematocrit of the unit stored at 4°C for 5 weeks with warming on Day 14/hematocrit of the unit stored at 4°C for 5 weeks with warming on Day 28/hematocrit of the unit stored at 4°C for 6 weeks (control unit).

more rapidly in the days after warming (Fig. 1C). Mean RBC ATP concentrations in the warmed cells at 30 days of storage were approximately equal to those of the conventionally stored cells after 42 days. RBC ATP concentrations at the end of storage, 3.4 ± 0.2, 2.9 ± 0.2, and 2.8 ± 0.2 µmol per g of Hb in the control units, the test units warmed on Day 14, and the test units warmed on Day 28, respectively, were not significantly lower in the warmed units ($p = 0.077$), but varied in a manner that was highly donor-specific (Fig. 2A, $p = 0.007$). RBC ATP concentrations at the beginning of storage did not predict concentrations at the end of storage.

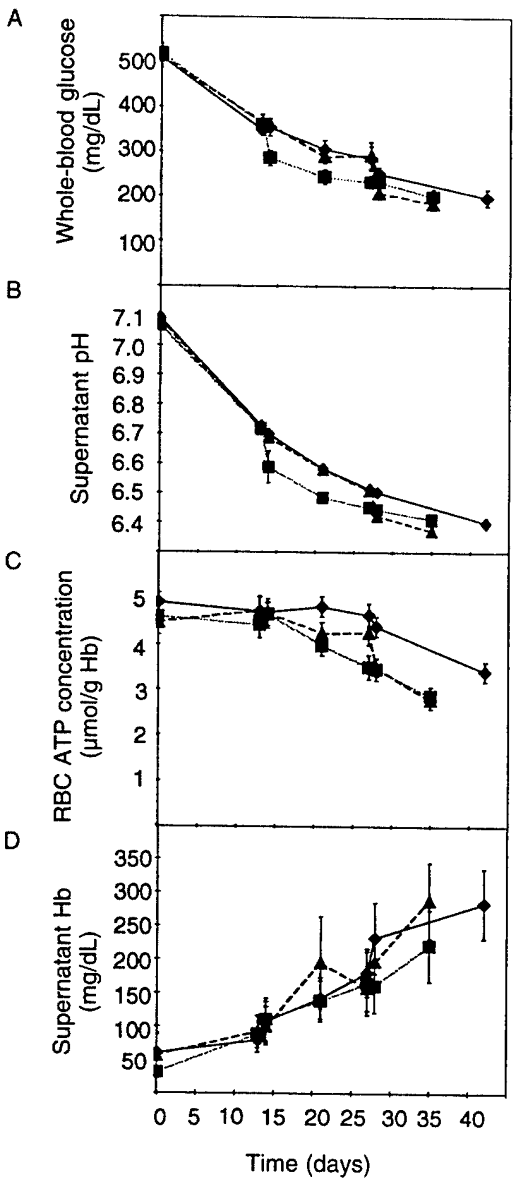

Fig. 1. Measures (mean ± SEM, n = 11 at all points) of A) whole-blood glucose concentration, B) supernatant pH, C) RBC ATP concentration, and D) supernatant Hb concentration. The three arms of the study are 1) 4°C storage for 6 weeks (–◆–), 2) 4°C storage for 5 weeks with warming to 25°C on Day 14 (···■···), and 3) 4°C storage for 5 weeks with warming to 25°C on Day 28 (--▲--). The decrease in pH corresponds to the consumption of glucose, and both are accelerated by warming. RBC ATP concentration decreases more rapidly after warming, presumably because ATP synthesis is slower at reduced pH. RBC lysis measured as supernatant Hb concentration increases with storage time but never reaches a value of 520 mg per dL, equivalent to lysis of 1 percent of the cells.

Lack of bacterial contamination

Three to 4 days before the end of the assigned storage period, all units were cultured for bacteria; none showed evi-

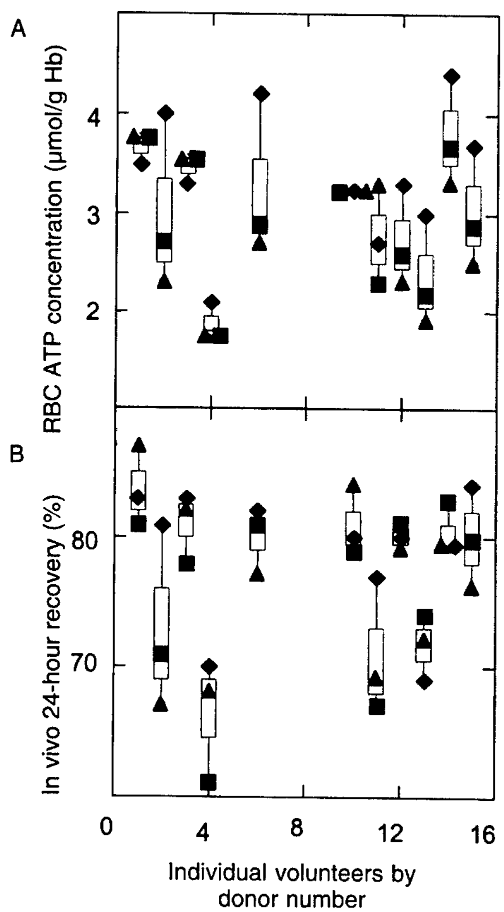

Fig. 2. Individual measurements of A) RBC ATP concentration at the end of storage and B) single-label 24-hour RBC recovery in the individual donors. The three arms of the study are 1) 4°C storage for 6 weeks (◆), 2) 4°C storage for 5 weeks with warming to 25°C on Day 14 (■), and 3) 4°C storage for 5 weeks with warming to 25°C on Day 28 (▲). RBC ATP concentration at the end of storage is correlated with viability, but it has little discriminant value for choosing units that store well. The poor survival of RBCs from a few donors represents the greatest source of variability in this study. The variability in measurements of RBC volume with ^{99m}Tc in the same donor make the double-label survival measurements less precise.

dence of contamination or growth. At the end of the assigned storage period, the mean fraction of hemolyzed RBCs was less than 1 percent under all conditions of storage (Fig. 1D).

RBC recovery

The measured recoveries of RBCs returned to original donors at the end of storage were not statistically different in the three storage groups. This was true whether recovery was measured by the ^{51}Cr single-label method or by the $^{99m}Tc/^{51}Cr$ double-label method (Fig. 3). The values for the single-label method were 79 ± 2 percent for cells stored at 4°C for 6 weeks, 76 ± 2 percent for cells warmed on Day 14, and 76 ± 2 percent for cells warmed on Day 28. The double-label method gave values that averaged 2.5 percent lower. The mean values were 78 ± 3 percent, 75 ± 2 percent, and 72 ± 2 percent for the cells stored at 4°C for 6 weeks, for the cells warmed on Day 14, and for the cells warmed on Day 28, respectively. Although the recovery percentages of the warmed RBCs are lower on average, this difference was not significant because of the large variability of recoveries under all conditions among the individual volunteers. This difference among donors is best seen in the single-label measurements (Fig. 2B, $p = 0.003$). The double-label recovery measurements were performed to correct for the systematic error caused by the early and unmeasured loss of ^{51}Cr-labeled stored cells in the first 5 minutes after return but before the start of mixing and sampling. The three measures of ^{99m}Tc RBC mass in the individual volunteers were reproducible to 5 ± 3 percent with a range of 1 to 10 percent. Thus, the double-label recovery measurements correct a 2.5-percent systematic error, but in doing so, they induce an additional 5-percent random error because of the inherent stochastic errors of radiation counting and microhematocrit measurement. For analysis within a group, the greater precision of the individual single-label measures brings out correlations. For the comparison of average viability, the means of groups measured with the double-label method are more accurate.

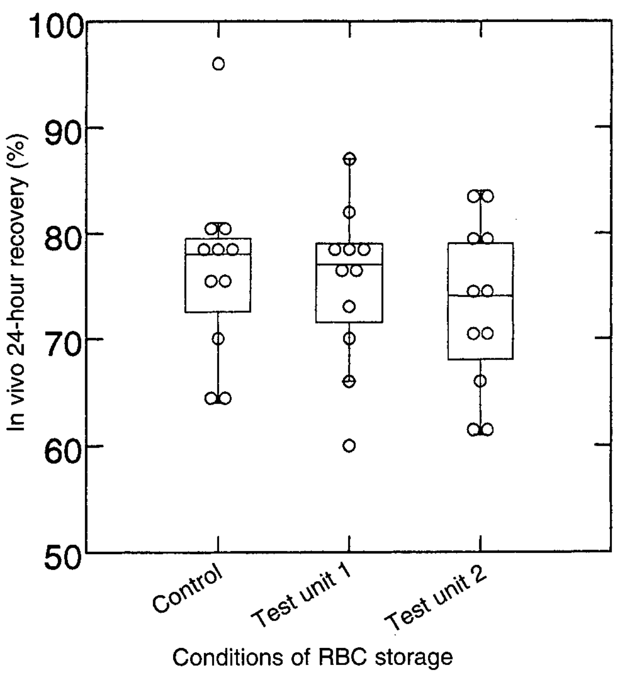

Fig. 3. Viability measured as the 24-hour in vivo RBC recovery using the $^{99m}Tc/^{51}Cr$ double-label method did not vary under the different conditions of storage. Control = units stored at 4°C for 6 weeks, test unit 1 = units stored at 4°C for 5 weeks with 25°C storage on Day 14, and test unit 2 = units stored at 4°C for 5 weeks with 25°C storage on Day 28.

RBC survival

The viable mass of returned RBCs that were circulating 24 hours after the return declined at about 1.4 percent per day during the next 2 weeks in all volunteers and under all conditions of storage. At 7 days, the fractional survival averaged 89 ± 1 percent; at 14 days, it was 81 ± 1 percent.

Correlation between RBC ATP concentration and RBC recovery

The RBC recovery measured by either the single- or double-label method correlated with the RBC ATP concentration with the correlation coefficient $n = 0.627$ ($p = 0.001$) for the single-label method and $r = 0.496$ ($p = 0.003$) for the double-label method. The fraction of the total variability in RBC recovery explained by the correlation with ATP concentration (the coefficient of variation [r^2]) was 0.393 and 0.246 for the single- and double-label methods, respectively. Because the range of errors induced by the double-label method was almost as large as the variability in measured recovery, it reduced r^2 by almost one-half.

The relationship between the slope of the regression line for RBC recovery on RBC ATP concentration was an 8-percent decrease in viability (RBC recovery) with each µmol decrease per g of Hb in RBC ATP concentration. The lower RBC ATP concentration observed in the warmed units at the end of storage (0.55 µmol/g Hb) predicts almost exactly the 4.5-percent mean decrease in observed RBC recovery. The slope of the decrease in RBC ATP concentration in the last weeks of storage seen in Fig. 1C (0.7 µmol/g Hb/week) makes this difference equivalent to 5 fewer days of storage.

DISCUSSION

This study shows that RBCs stored in AS-5 and warmed to 25°C for 24 hours on either Day 14 or 28 in the course of 35 days of 4°C storage have an in vivo recovery and survival that are not statistically different from those of RBCs stored at 4°C for 42 days. Nor do they display excessive hemolysis.

The decrease in viability associated with warming seems to be caused by the temperature-related increased rate of metabolism and the resulting earlier onset of reduced pH and conditions that adversely affect the maintenance of RBC ATP concentrations. Three separate measures—the rates of glucose consumption, the times when RBC ATP concentrations are equal, and the correlation of rates of decline in RBC ATP concentrations with RBC recovery—converge to suggest that one day of storage at 25°C reduces the storage time by 12 days. Thus, RBCs transfused after refrigerated storage for 29 days and one day at 25°C are expected to display the required 75-percent mean viability with less than 1-percent hemolysis.[10] Further, because the decrease in RBC ATP concentrations in the later weeks of RBC storage is linear with time, a 2-hour exposure at 25°C probably shortens the time to unacceptable RBC recovery by a day. However, as Shields[4] noted, such short exposures to higher temperatures produce differences in RBC recovery that are too small to measure.

It seems likely that the effects of temperature on RBC recovery that were measured here during storage in AS-5 also apply to the other additive solutions with the same volumes and similar composition: saline-adenine-glucose-mannitol and AS-1 (Adsol, Baxter Healthcare, Roundlake, IL). We previously reported measures of RBC ATP concentrations after normal and warm storage of RBCs in CPDA-1 and showed a quantitatively similar decrease.[12] AS-3 (Nutricel, Medsep Corp., Covina, CA) shows a similar decrease in RBC ATP concentrations with time in the cold, but no data exist on its performance with warming.

According to the first edition of what is now called the *Technical Manual*, "When blood is issued for transfusion, it must be given to the patient within a reasonable period of time or promptly returned to the blood bank. If the blood has been beyond the direct control of the blood bank personnel for 24 hours or more, no responsibility can be taken for its safety or quality..."[13(p 71)] This statement reflected a general understanding of the temperature-dependent rates of RBC metabolism and bacteria growth. Now, the standards[1] of the American Association of Blood Banks and FDA regulations state that RBCs may not be issued unless they have been stored at temperatures between 1 and 6°C and shipped at temperatures between 1 and 10°C. The regulation has been widely interpreted to mean that RBCs that have spent more than 30 minutes out of the refrigerator, and therefore might have warmed above 10°C, should be destroyed.[14] The actual data on which this "30-minute rule" was based showed that units of whole blood in plastic bags warmed to temperatures above 10°C in 45 to 60 minutes after removal from blood bank refrigerators.[15] In the ninth edition of the *Technical Manual*, the justification for the rule was stated: "Warming the blood beyond...(10°C), even with subsequent cooling, tends to accelerate red cell metabolism, produce hemolysis, and may permit bacterial growth in the unit."[16(p 49-50)] Bacteria growth in warmed units of RBCs is rare.[17]

The 30-minute rule is a measurable and enforceable quality control standard. However, it is wasteful of blood and often limits the availability of blood at sites of critical care. Surgeons rightfully complain. The 30-minute rule should be reconsidered in the light of present knowledge and should be dealt with explicitly in future FDA regulations and American Association of Blood Banks standards. In the past, the FDA has not allowed alternative procedures for RBC storage under 21 CFR 640.120 where the temperature of RBCs exceeded 10°C. In the future, a revised 30-minute rule should lead to an evidence-based decision regarding the safety and viability of the units in question, not to an automatic requirement for destruction. Such a decision should be made within a framework of revised government regulations, accrediting agency standards, and facility guidelines by a technologist or the medical director.

In the meantime, these data probably have use only for medical directors making critical decisions about the safety of units accidentally exposed to room temperatures in the course of emergency situations, when other blood of known safety and viability is not available. The present standards provide excellent protection for the viability and bacteriologic safety of RBCs and should not be violated except in true emergencies.

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Appendix M - (The effects of polyvinyl chloride and polyolefin blood bags on red blood cells stored in a new additive solution)

Vox Sanguinis (2001) **81**, 161–166

ORIGINAL PAPER

The effects of polyvinyl chloride and polyolefin blood bags on red blood cells stored in a new additive solution

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Vox Sanguinis

Background and Objectives Red blood cells (RBCs) must be stored in polyvinyl chloride (PVC) bags plasticized with di-2-ethylhexyl phthalate or a similar plasticizer to achieve their full storage life with conventional storage solutions. Improved storage solutions might remove this requirement and allow blood storage in other plastics. Experimental Additive Solution-61 (EAS-61), which maintains RBCs for 9 weeks with reduced haemolysis and satisfactory ^{51}Cr 24-h recovery, is an appropriate candidate improved RBC storage solution.

Materials and Methods Twenty-four units of packed RBCs were pooled in groups of four units, each pool was realiquoted into four units and stored, six pooled units per arm, in one of the following: 100 ml of EAS-61 in PVC; 200 ml of EAS-61 in PVC; 100 ml of EAS-61 in polyolefin (PO); and 200 ml of EAS-61 in PO. Haemolysis, RBC morphology indices, RBC ATP concentrations, and other measures of RBC metabolism and function were measured weekly.

Results RBC haemolysis exceeded 1% by 7 weeks in PO bags containing 100 ml or 200 ml of EAS-61. In PVC bags, haemolysis was less than 1% at 11 weeks. RBC ATP concentrations were 1 mol/g of haemoglobin (Hb) higher at 2 weeks in the PVC-stored units.

Conclusions RBCs stored in PVC had markedly less haemolysis and higher RBC ATP concentrations than those stored in PO. Haemolysis would limit RBC storage in PO bags to a duration of 6 weeks, even with EAS-61.

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Key words: blood storage, di-2-ethylhexyl phthalate, humans, plastics, polyolefin, polyvinyl chloride, RBC ATP concentrations, RBC haemolysis, RBC storage.

Introduction

Plastic blood bags make transfusion safer for patients, make blood handling safer for health care workers, and reduce the burden of medical waste on the environment [1]. However, plasticizers can leach from the plastic bags into the contained fluids [2], bag makers can be exposed to carcinogenic vinyl chloride monomer [3], and the ozone layer can be degraded by incineration of medical wastes containing chlorinated hydrocarbons [4]. Alternatives to the presently used blood bag plastics might retain the healthcare benefits of plastic blood bags while avoiding some of their environmental consequences.

Polyvinyl chloride (PVC) plasticized with di-2-ethylhexyl phthalate (DEHP) is the standard material used for red blood cell (RBC) storage bags. This plastic was chosen initially for its manufacturing and handling qualities [5]. It was subsequently shown that the DEHP improves RBC storage by reducing haemolysis and membrane loss by microvesiculation [6,7]. Other plastics, such as polyolefin (PO), which is used in platelet storage bags because of its high gas permeability, were

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associated with greater RBC haemolysis and microvesiculation when used in conjunction with standard storage solutions, but performed better when DEHP or similar plasticizers were added to the stored RBCs [8–10]. Without the protection afforded by the plasticizer, RBC storage would be limited to 3 weeks with conventional storage solutions [11,12].

Recently, we have developed an additive storage solution, Experimental Additive Solution-61 (EAS-61), in which RBCs can be stored for 9 weeks in PVC bags [13]. EAS-61 works, in part, by suppressing RBC microvesiculation and haemolysis. The increased volume of additive solution also seemed to be important for raising RBC ATP concentrations, improving RBC morphology and reducing haemolysis [14]. To evaluate whether EAS-61 might suppress RBC haemolysis during storage in a different plastic and to learn more about cellular mechanisms important for blood storage, we conducted a study comparing RBCs stored in PVC and PO containers in 100- and 200-ml volumes of EAS-61. Although haemolysis of RBCs stored in EAS-61 was below conventional limits for at least 6 weeks, RBCs stored in PO bags had four times more haemolysis and lower RBC ATP concentrations than those stored in PVC.

Materials and methods

Volunteers

Twenty-four healthy volunteers, who were donating full units of blood for the collection of anthrax-immune plasma, gave consent for their RBCs to be used in storage studies. The donors met standard blood donor criteria and gave informed consent in accordance with protocols approved by the Institutional Review Board of the Walter Reed Army Institute of Research and the U.S. Army's Human Subjects Research Review Board.

Storage solution

The EAS-61 solution (composition: NaCl 26 mM, adenine 2 mM, glucose 110 mM, mannitol 55 mM, Na_2HPO_4 12 mM) was prepared in the laboratory from high-purity adenine, sugars, salts and water, and sterile-filtered into 1-l PO storage bags (code 4R2238; Baxter Healthcare Corp., Roundlake, IL). The bags were stored at 37 °C for 2 weeks. On the eighth day, a sample was sent to a commercial reference laboratory (The Associates of Cape Cod, Falmouth, MA) for endotoxin testing using the Limulus Amoebocyte Lysate assay. At the end of 2 weeks, the solutions were cultured. The cultures were incubated for a further 2 weeks. Sterility was confirmed by the absence of bacterial and fungal growth in the cultures after 14 days.

Study design

We conducted a 'pooling' study to evaluate RBC morphology, metabolism and integrity over the course of 11 weeks of storage in PVC and PO bags. Pooling reduces the largest source of variability in conventional blood storage studies, i.e. the differences between RBCs from different donors [15], by placing some of the cells from each donor in every treatment group of the study while maintaining conventional unit size. Twenty-four RBC units, unreactive in the indirect antiglobulin test (IAT), were grouped into sets of four ABO-matched units. Each set was then pooled, mixed and aliquoted by weight to make four pooled units. Blood was handled in a manner intended to minimize contact with PVC plastic until the pooled RBCs were aliquoted.

The four units from each pool were aliquoted as follows: two units into PO bags and two units into PVC bags. The two units in each pair were then diluted, one with 100 ml of EAS-61 and the other with 200 ml of EAS-61. The units were stored at 1–6 °C for 11 weeks. Electrolytes, pH, metabolites, blood gases, RBC morphology indices, haemolysis, and RBC ATP concentrations were measured weekly.

RBC unit preparation

Standard units (450 ± 45 ml) of blood were collected from each donor into 63 ml of citrate-phosphate-dextrose (CPD) anticoagulant in 1-l PO bags (4R2238, Baxter Healthcare Corp.). The bags had been modified by sterile transfer of the CPD and the needle with a short length of the PVC tubing from a commercial triple bag collection set (BB*AGD456A; Terumo Medical Corp, Elkton, MD) using a sterile connecting device (SCD 312; Terumo Medical Corp.). Twenty of the units were drawn on a mobile blood drive and refrigerated at 4 °C for 1–6 h before transportation in a shipping container with wet ice 40 miles to the laboratory. Four units were drawn in the laboratory and processed within 4 h. Packed cells were prepared by centrifugation (at 5000 *g*) for 5 min at 4 °C followed by the removal of sufficient plasma to achieve a haematocrit of 85%. ABO/Rh and IAT testing were performed using tube methods and commercial reagents (Immucor, Inc., Norcross, GA). ABO-matched, IAT non-reactive RBCs were then pooled separately, in groups of four, in six one-litre PO bags, mixed thoroughly, and aliquoted into the study units by weight using sterile tubing connection for all transfers. The aliquots were transferred to either 600-ml PVC transfer bags (code 4R2023; Baxter Healthcare Corp.) or 1-l PO storage bags (code 4R2238, Baxter Healthcare Corp). Either 100 ml or 200 ml of EAS-61 was added to the RBC aliquot prior to storage.

In vitro measurements

Samples were collected, after gentle mixing by inversion, by a sterile sampling procedure in a biological safety cabinet. A battery of *in vitro* tests was performed on all units at the beginning of storage and weekly thereafter.

The total haemoglobin (Hb) concentration was measured by using a clinical haematology analyser (Hematology Cell Counter System Series 9110+; Baker, Allentown, PA). Supernatant Hb was measured spectrophotometrically using the modified Drabkin assay [16]. Percentage haemolysis was determined by the ratio of free to total haemoglobin. The results are expressed as percentage haemolysis to compensate for the differences in haematocrit and Hb concentrations between samples. Centrifuged microhaematocrits (Clay Adams; Becton-Dickinson, Rutherford, NJ) were performed to avoid the readjustment of cell volume and subsequent errors caused by the isotonic diluent used in the blood analysers.

RBC ATP, glucose and lactate concentrations were measured in supernatants of deproteinized RBCs. Cell aliquots were mixed with cold 10% trichloroacetic acid to precipitate blood proteins, centrifuged at 2700 g for 10 min, and the protein-free supernatant was frozen at – 80 °C until tested. ATP was assayed enzymatically using a commercially available test kit (Procedure 366-UV; Sigma Diagnostics, St Louis, MO).

The concentration of supernatant chloride, pH and blood gases were measured using a blood gas analyser (Corning 855; Corning, Ithaca, NY). Thus, pH was measured at 37 °C. Sodium, potassium, phosphate, lactate and glucose were measured on a programmable chemical analyser (Hitachi 902 Analyser; Boehringer Mannheim Corporation, Indianapolis, IN). The average degree of RBC shape change, from discocytes to echinocytes to spherocytes, was measured according to the method of Usry *et al.* [17].

Statistical analysis

Comparisons of means of measured values at given times between the arms of the trial were evaluated by analysis of variance. P-values of $< 0{\cdot}05$ were considered statistically significant.

Results

Significant differences were observed between the PVC- and PO-stored cells in RBC ATP concentrations, RBC morphology and haemolysis. Figure 1(a) and Table 1 show that in the early phases of storage, RBC ATP concentrations were, on average, 1 µmol/g of Hb higher in the PVC-stored cells. Initially, the RBC ATP concentration increased in the PVC-stored cells, but not in the PO-stored RBC. At the end of storage, this difference diminished as the RBC ATP concentration decreased more slowly in the PO-stored cells. This difference was not related to differences in the rate of glycolysis (data not shown) or increasing lactate concentrations (Fig. 1b), which were the same for the equivalent volumes of EAS-61. RBC morphology indices were worse in PO-stored cells after the first week of storage (Fig. 1c). The rate of decline in the morphology indices was twice as great in PO than in PVC.

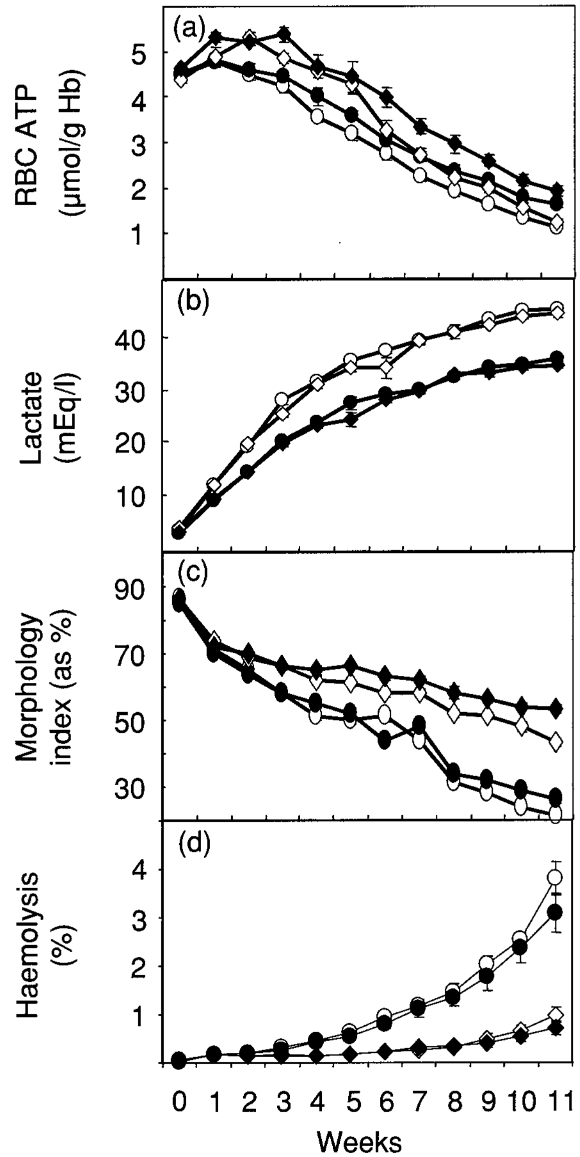

Fig. 1 Weekly measurements of (a) red blood cell (RBC) ATP concentration; (b) RBC lactate concentration; (c) RBC morphologic index; and (d) fractional RBC haemolysis for the RBCs stored in: 100 ml of Experimental Additive Solution-61 (EAS-61) in polyvinyl chloride (PVC) (◇); 200 ml of EAS-61 in PVC (◆); 100 ml of EAS-61 in polyolefin (PO) (○); and 200 ml of EAS-61 in PO (●). RBC ATP concentrations were higher in the early phases of storage in PVC bags, but the amount of lactate produced did not differ. RBCs stored in PO bags showed greater morphological change and haemolysis. Data are presented as mean ± standard error of the mean (n = 6 pooled units per group).

There was a small (but not statistically significant) effect of increased storage volume, leading to slightly better morphology (Table 1). Finally, RBC stored in PO had four times the haemolysis of cells stored in PVC (Fig. 1d). Increasing the volume of EAS-61 from 100 ml to 200 ml had a small (but

Table 1 Selected values of red blood cell (RBC) ATP concentration, haemolysis and morphology during storage

Plastic and volume of EAS-61	RBC ATP (mol/g of Hb)		Haemolysis (%)			Morphology index	
	2 weeks	9 weeks	6 weeks	7 weeks	9 weeks	7 weeks	9 weeks
PVC with 100 ml	5·32 ± 0·11	1·97 ± 0·10	0·24 ± 0·03	0·25 ± 0·02	0·49 ± 0·11	58 ± 1·2	51 ± 1·3
PVC with 200 ml	5·22 ± 0·10	2·58 ± 0·13	0·24 ± 0·02	0·31 ± 0·04	0·39 ± 0·08	62 ± 1·0	56 ± 1·0
PO with 100 ml	4·47 ± 0·08	1·62 ± 0·06	0·93 ± 0·09	1·16 ± 0·12	2·03 ± 0·17	44 ± 1·4	28 ± 0·36
PO with 200 ml	4·60 ± 0·14	2·15 ± 0·08	0·80 ± 0·08	1·12 ± 0·19	1·76 ± 0·27	48 ± 1·4	32 ± 0·41

EAS-61, Experimental Additive Solution-61; Hb, haemoglobin; PO, polyolefin; PVC, polyvinyl chloride.

again not statistically significant) effect on reducing haemolysis in this system (Table 1). RBC haemolysis exceeded 0·8% by 6 weeks and 1% by 7 weeks in PO bags containing 100 ml or 200 ml of EAS-61.

Gas transport was also different in the two kinds of bags, but it did not appreciably affect the storage solution pH (Fig. 2a). The partial pressure of CO_2 (Fig. 2b) and the concentration of bicarbonate (Fig. 2c) were higher in the PVC bags after the first week. The oxygen partial pressure in the PO bags was also higher at all time-points after the initial measurement (Fig. 2d). The shoulder of the oxygen saturation curve was reached at 3 weeks in PO and at 10 weeks in PVC.

Electrolytes were not affected by storage bag composition. Supernatant sodium concentration decreased (Fig. 3a), and potassium concentration increased (Fig. 3b), with storage. The chloride concentration of the suspending fluid (Fig. 3c) increased for the first 2 weeks and then slowly decreased. Phosphate concentration in the suspending solution (Fig. 3d) decreased in the first 2 weeks of storage and slowly increased thereafter. The sum of the concentrations of the cations, sodium and potassium, and the anions, chloride, bicarbonate, phosphate and lactate, were approximately equal when corrected for valence.

Discussion

This study showed that EAS-61, a new RBC storage solution, could potentially increase the shelf-life of RBC stored in PO bags to 5 or 6 weeks. However, RBC stored in PO bags had four times the amount of haemolysis at each weekly measurement as when the same cohort of cells were stored in PVC bags. RBCs that had a clinically acceptable fractional haemolysis of less than 0·8% after 10 weeks when stored in PVC bags, had greater than 0·8% haemolysis at 6 weeks and greater than 1% haemolysis at 7 weeks when stored in PO bags in EAS-61.

The increased haemolysis was probably related to the absence of DEHP in the PO bags. Greenwalt and his colleagues have shown that RBCs stored in CPD in PO bags have more rapid shape change, greater membrane loss and increased haemolysis [10]. This haemolysis was mostly in the form of shed microvesicles. When they added DEHP to RBC stored in the PO bags, the shape change, microvesiculation and haemolysis were reduced. In the present study, exposure of warm blood to PO bags for just a few hours in the course of pooling caused a rapid initial shape change which persisted but progressed at a slower rate after the RBCs were transferred to PVC bags. We have previously reported that RBCs stored continuously in PVC bags in 100 ml or 200 ml of EAS-61 had a more discoid morphology throughout storage than was observed in this study, yet very similar rates of haemolysis with time [14]. Therefore, it appears that DEHP can suppress further shape change and haemolysis, even after the shape change process has been initiated.

RBC ATP concentrations were lower in the PO-stored cells. However, the rates of glucose consumption and lactate formation were the same for RBC stored in both types of plastic bags. This means that overall rates of glycolysis were the same. In the Embden-Meyerhof pathway, 1 mole of lactate is produced for every mole of ATP produced, so the rates of ATP synthesis might be expected to be similar. However, the RBC ATP concentration differences, about 0·8 μmol/g of Hb at 2 weeks, represent the product of only 1% of total glycolysis based on the amount of lactate produced by that time, and thus no useful relationship can be elucidated. The concentrations of RBC ATP are a function of the rates of synthesis and the rates of utilization. RBC ATP was either made less efficiently or was consumed at a greater rate in a process that was temporally related to the RBC shape changes.

The PO bags have greater gas permeability than the PVC bags. As a result, CO_2 partial pressures were lower and O_2 partial pressures were higher in the PO bags throughout the storage interval. These differences in gas tension may have contributed to the RBC ATP differences in two different ways. First, the loss of CO_2 might have affected the intracellular pH through the bicarbonate buffer system. Second, the higher partial pressure of O_2 may have led to oxidative stress. Significant consequences of either of these effects seem unlikely based on the work of Greenwalt and his colleagues cited above, which showed that adding DEHP to RBC in PO bags improved RBC ATP concentrations and reduced haemolysis.

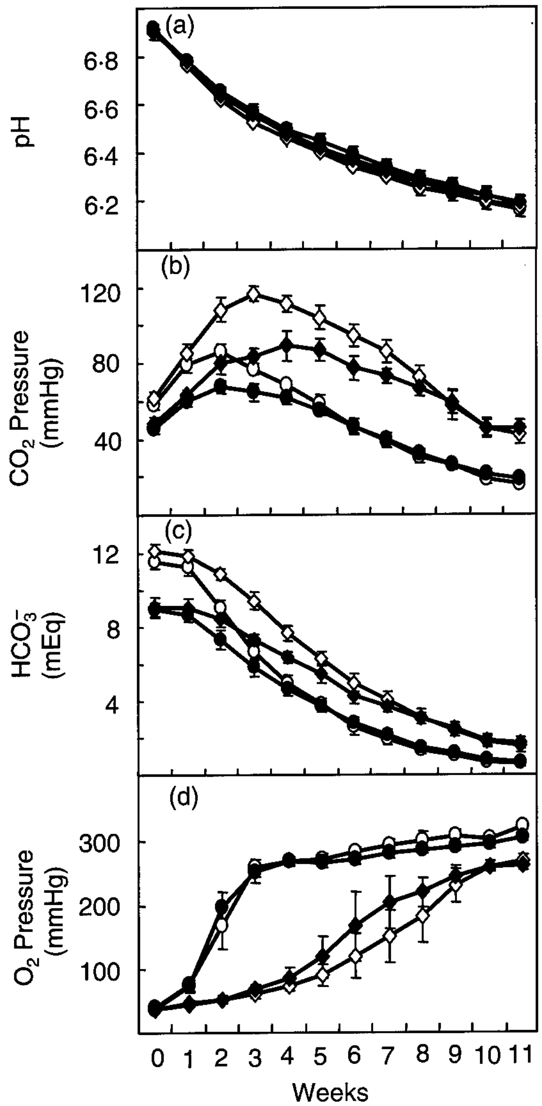

Fig. 2 Weekly measurements of (a) supernatant pH; (b) CO_2 partial pressure; (c) bicarbonate concentration; and (d) oxygen partial pressure for the red blood cells (RBCs) stored in: 100 ml of Experimental Additive Solution-61 (EAS-61) in polyvinyl chloride (PVC) (◇); 200 ml of EAS-61 in PVC (◆); 100 ml of EAS-61 in polyolefin (PO) (○); and 200 ml of EAS-61 in PO (●). Diffusion of CO_2 out of and of O_2 into the PO bags was ≈ three times more rapid than observed with PVC bags, but there was no significant effect on pH. Data are presented as mean ± standard error of the mean (n = 6 pooled units per group).

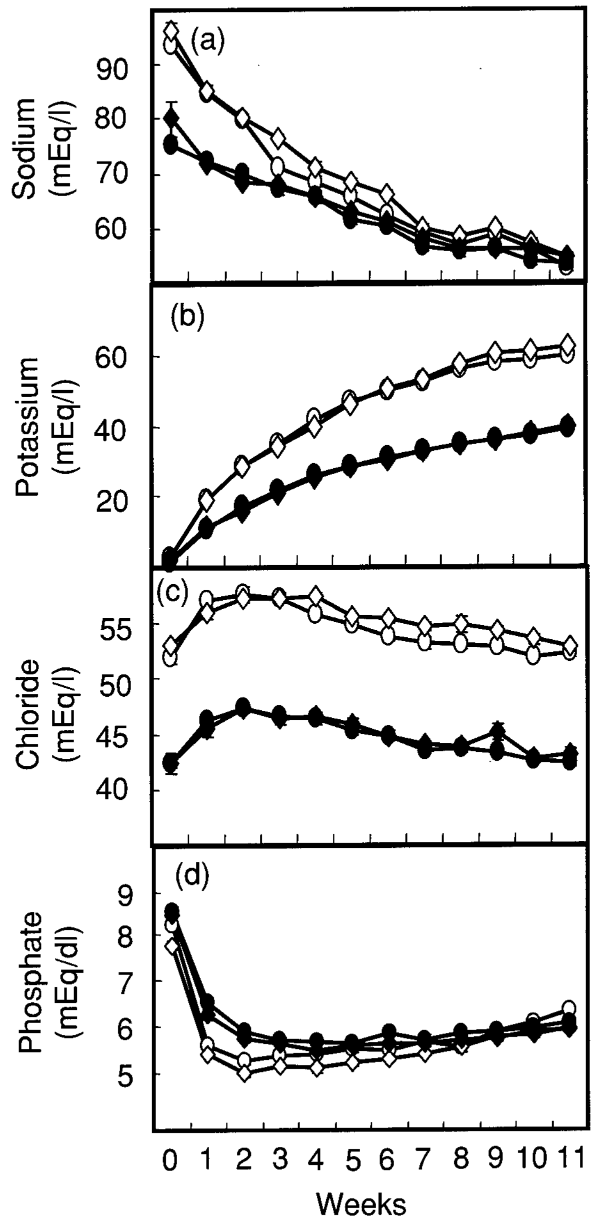

Fig. 3 Weekly measurements of (a) supernatant sodium concentration; (b) supernatant potassium concentration; (c) supernatant chloride concentration; and (d) supernatant inorganic phosphate concentration for the red blood cells (RBCs) stored in: 100 ml of Experimental Additive Solution-61 (EAS-61) in polyvinyl chloride (PVC) (◇); 200 ml of EAS-61 in PVC (◆); 100 ml of EAS-61 in polyolefin (PO) (○); and 200 ml of EAS-61 in PO (●). EAS-61 volume, but not bag composition, affected the electrolyte composition. Data are presented as mean ± standard error of the mean (n = 6 pooled units per group).

Hogman and his colleagues suggested that the lack of DEHP may also contribute to the increased microvesiculation and haemolyis that occurs when RBC are stored tightly packed and unmixed in PVC bags [18]. Reduced RBC ATP concentrations were also observed in these densely packed and unmixed cells, but were attributed to locally low pH.

Recently, efforts to understand the signal transduction of microvesiculation or 'bleb' formation in apoptotic cells has led to the identification of a signal transduction pathway wherein caspase-3 cleaves the ROCK-1 kinase to produce a truncated molecule with constitutive myosin light-chain kinase activity [19,20]. Activation of the mechanism leads to cytoskeletal contraction and bleb formation. Knock-out deletions of ROCK-1 prevent bleb formation without preventing other aspects of apoptosis. Such a mechanism could tie together the major findings of this study: reduced RBC ATP and increased shape change and haemolysis. It may provide a basis for understanding the effects of DEHP and for understanding significant events in the development of the RBC storage lesion.

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Appendix N - (A multicenter study of in vitro and in vivo values in human RBC's frozen with 40-percent (wt/vol) glycerol and stored after glycerolization for 15 days at 4°C in AS-3; assessment of RBC processing in the ACP 215)

A multicenter study of in vitro and in vivo values in human RBCs frozen with 40-percent (wt/vol) glycerol and stored after deglycerolization for 15 days at 4°C in AS-3: assessment of RBC processing in the ACP 215

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BACKGROUND: The FDA has approved the storage of frozen RBCs at –80°C for 10 years. After deglycerolization, the RBCs can be stored at 4°C for no more than 24 hours, because open systems are currently being used. Five laboratoris have been evaluating an automated, functionally closed system (ACP 215, Haemonetics) for both the glycerolization and deglycerolization processes.
STUDY DESIGN AND METHODS: Studies were performed at three military sites and two civilian sites. Each site performed in vitro testing of 20 units of RBCs. In addition, one military site and two civilian sites conducted autologous transfusion studies on ten units of previously frozen, deglycerolized RBCs that had been stored at 4°C in AS-3 for 15 days. At one of the civilian sites, 10 volunteers received autologous transfusions on two occasions in a randomized manner, once with previously frozen RBCs that had been stored at 4°C in AS-3 for 15 days after deglycerolization and once with liquid-preserved RBCs that had been stored at 4°C in AS-1 for 42 days.
RESULTS: The mean ± SD in vitro freeze-thaw-wash recovery value was 87 ± 5 percent; the mean ± SD supernatant osmolality on the day of deglycerolization was 297 ± 5 mOsm per kg of H_2O, and the mean ± SD percentage of hemolysis after storage at 4°C in AS-3 for 15 days was 0.60 ± 0.2 percent. The paired data from the study of 10 persons at the civilian site showed a mean ± SD 24-hour posttransfusion survival of 76 ± 6 percent for RBCs that had been stored at 4°C for 15 days after deglycerolization and 72 ± 5 percent for RBCs stored at 4°C in AS-1 for 42 days. At the three sites at which 24-hour posttransfusion survival values were measured by three double-label procedures, a mean ± SD 24-hour posttransfusion survival of 77 ± 9 percent was observed for 36 autologous transfusions to 12 females and 24 males of previously frozen RBCs that had been stored at 4°C in AS-3 for 15 days after deglycerolization.
CONCLUSION: The multicenter study showed the acceptable quality of RBCs that were glycerolized and deglycerolized in the automated ACP 215 instrument and stored in AS-3 at 4°C for 15 days.

Previously frozen RBCs are FDA-approved for storage at –80°C for 10 years. After deglycerolization, the RBCs can be stored at 4°C for no more than 24 hours, because the current methods for glycerolization and deglycerolization are open systems with the potential for bacteriologic contamination. The instrument used in this study is an automated, functionally closed system for the glycerolization and deglycerolization of human RBCs (ACP 215, Haemonetics Corp., Braintree, MA) that uses a sterile connector device (SCD, Haemonetics), inline 0.22-µ filters to deliver solutions, a disposable polycarbonate bowl with an external seal, and an integrally attached shaker and a printer to record the glycerolization and deglycerolization processes. The quality of the RBCs is monitored by an optical system that measures the Hb concentration in the waste solution during the deglycerolization

ABBREVIATIONS: FT = freeze-thaw; FTW = freeze-thaw-wash; NBRL = Naval Blood Research Laboratory; NMC = Naval Medical Center; SCD = sterile connector device; UMASS = University of Massachusetts Memorial Health Care; WRAIR = Walter Reed Army Institute of Research.

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procedure. This multicenter study was done to assess the quality of RBCs glycerolized and deglycerolized in the automated ACP 215.

Studies at the five sites were conducted over a 1-year period. The three military locations were Walter Reed Army Institute of Research (WRAIR, Silver Spring, MD), the Naval Medical Center Portsmouth (NMC Portsmouth), Portsmouth, VA), and the Naval Medical Center Great Lakes (NMC Great Lakes, Great Lakes, IL). The two civilian sites were the Naval Blood Research Laboratory (NBRL, Boston University School of Medicine, Boston, MA), and the University of Massachusetts Memorial Health Care (UMASS, Worcester, MA). At each of the five sites, in vitro studies were done on 17 to 28 units of RBCs. About 10 autologous transfusions were performed at each of the two civilian locations (NBRL and UMASS) and at one military location (WRAIR), and 24-hour posttransfusion survivals were measured with double-isotope procedures. The NBRL also conducted a randomized study in which each of 10 volunteers received an autologous transfusion on two separate occasions, once with previously frozen, deglycerolized RBCs that had been stored at 4°C in AS-3 for 15 days and a second time with liquid-preserved RBCs that had been stored at 4°C in AS-1 for 42 days.

MATERIALS AND METHODS

One hundred sixty-five (165) healthy male and female volunteers who met the requirements of the AABB for acceptable blood donors each donated a unit of whole blood for in vitro studies. Twelve of the female volunteers and 24 of the male volunteers also participated in autologous transfusion studies.

The study was reviewed and approved by the Institutional Review Board for Human Research (Boston University Medical Center, Boston, MA); the Human Use Review Committee, WRAIR; the Clinical Investigation and Research Department, NMC Portsmouth; the Clinical Investigation and Research Department, NMC Great Lakes; and the Committee for the Protection of Human Subjects in Research, UMASS. Informed consent was obtained from each volunteer.

From volunteers who had a Hct value of at least 40 percent and no more than 46 percent, 450 g of whole blood was collected. From volunteers whose Hct values were greater than 46 percent, 430 g of blood was collected. The blood was collected into 63 mL of CPDA-1 in the 800-mL PVC plastic bag at a ratio of 7 volumes of blood to 1 volume of CPDA-1. The volume of RBCs collected was not greater than 180 mL, because this is the maximum volume that can be deglycerolized in the 275-mL disposable bowl used in the ACP 215 instrument.

The CPDA-1 whole blood was stored for up to 24 hours at 4°C, after which it was centrifuged at 1615 × *g* for 4 minutes, and the plasma was removed to produce a Hct of 75 ± 5 percent. The RBCs were stored at 4°C for a total of 6 days before glycerolization and freezing. With the use of a disposable glycerolization harness with an inline 0.22-μ filter in the ACP 215, the RBCs were glycerolized with a volume of 6.2 M glycerol solution to achieve a final glycerol concentration of 40 percent (wt/vol).

The glycerolized RBCs were centrifuged at 1250 × *g* for 10 minutes and the supernatant solution was removed.[1] The 800-mL PVC plastic bag containing the glycerolized RBC concentrate was overwrapped in a polyester plastic bag that was sealed and placed in a cardboard box. The glycerolized RBCs were frozen and stored in a –80°C mechanical freezer for at least 2 weeks.

The frozen RBCs were kept in the plastic overwrap to avoid bacterial contamination during thawing in the 42°C water bath (Blue-M, Lindberg, Asheville, NC) with a circulating pump. To reduce the thawing time when the 36°C water bath plasma thawer (Thermogenesis, Rancho Cordoba, CA) was used, the plastic bag overwrap was not used, because these RBCs were not directly exposed to water. After thawing to a surface temperature of 34°C, as monitored by an infrared scanner (Exergen Corp., Watertown, MA), the RBCs were deglycerolized with the SCD and the disposable set of the ACP 215. Each unit was deglycerolized in the instrument's disposable 275-mL blow-molded bowl with an external seal by the use of 50 mL of 12-percent NaCl, 1.6 L of 0.9-percent NaCl and 0.2-percent glucose, and 250 mL of AS-3 (Nutricel, Medsep Corp., Covina, CA). Each 100 mL of AS-3 contained 1.10 g of dextrose (monohydrate, USP), 0.588 g of sodium citrate (dihydrate, USP), 0.410 g of sodium chloride, 0.276 g of monobasic sodium phosphate (monohydrate), 0.042 g of citric acid (monohydrate), and 0.030 g of adenine.

The ACP 215 has a printer attached to the instrument to record the glycerolization and deglycerolization procedures. In addition, a graph records the hemolysis measured during the deglycerolization process. Total Hb concentration was measured by the cyanmethemoglobin method; the Hct was measured by the microhematocrit method; and supernatant Hb was measured by a spectrophotometric method (Spectronic Instruments, Syracuse, NY).[2] Osmolality was measured on a wide-range osmometer (Fiske Model 2400, Advanced Instruments, Norwood, MA), and intracellular and extracellular sodium and potassium ions were measured with a flame photometer (Model 943, Instrumentation Laboratory, Lexington, MA).[3] The percentage of hemolysis was calculated by dividing the total supernatant Hb by the sum of the total cellular Hb and total supernatant Hb. Bacterial cultures were performed on the previously frozen RBCs resuspended in AS-3 on the day of deglycerolization, after 15 days at 4°C, after 42 days at 4°C, and then after 30 days at 22°C. Cultures were also performed on the RBCs liquid-preserved in AS-1 (ADSOL, Baxter

Healthcare Corp, Deerfield, IL) after storage for 42 days at 4°C and again after 30 days at 22°C. In addition, both the previously frozen units and the liquid-stored units in the in vivo studies were sampled for culture 3 days before and on the day of the autologous transfusion. Cultures were done at both NBRL and the Microbiology Laboratory at UMASS. Aerobic blood agar and tryptic soy broth and anaerobic thioglycollate broth cultures were incubated at 37°C and examined for growth for 7 days.

Freeze-thaw-wash (FTW) recovery values were calculated by comparing the total Hb in the RBCs after thawing to the total cellular Hb after washing. RBC and platelet counts were determined on an automated counter (STKS, Coulter Corp., Hialeah, FL). WBC counts were performed with the STKS and the Nageotte hemocytometer with Turk's solution (Ricca Chemical Co., Arlington, TX), both on the day of deglycerolization and after storage at 4°C in AS-3 for 15 days.[4,5] Samples were diluted in Turk's solution and counted in a Nageotte chamber where 5 µL of neat sample was evaluated.

At each of the five sites, in vitro testing was done on about 20 units of previously frozen RBCs on the day of deglycerolization and after postwash storage at 4°C in AS-3 for 15 days. Measurements were made of freeze-thaw (FT) and FTW recovery values, Hb concentration, RBC count, platelet count, Hct value, supernatant Hb level, supernatant potassium level, and supernatant osmolality.

At NBRL, 14 volunteers received autologous transfusions of previously frozen RBCs that had been stored at 4°C in AS-3 for 15 days after deglycerolization. Ten of these 14 volunteers participated in a randomized study in which they also received autologous liquid-preserved RBCs stored at 4°C in AS-1 for 42 days. In this randomized study, the 5 µCi of ^{51}Cr and 0.5 µCi of ^{125}I albumin in the double-label procedure were used to measure the 24-hour posttransfu-sion survival.[6,7] Each volunteer's RBC volume was measured indirectly from the ^{125}I albumin plasma volume, and total body Hct was estimated from the peripheral venous Hct multiplied by 0.89. Blood samples were collected 5, 10, 15, 20, and 30 minutes and 24 hours after the autologous transfusion to measure the posttransfusion survival values of the ^{51}Cr-labeled previously frozen and liquid-preserved RBCs. The 100-percent survival value was calculated from the ^{51}Cr radioactivity associated with the transfused autologous RBCs and the recipient's RBC volume. The survival of the autologous preserved ^{51}Cr-labeled RBCs was assessed from the posttransfusion radioactivity as a percentage of the 100-percent survival value.[6]

In four females and four males studied at WRAIR who received autologous ^{51}Cr-labeled RBCs that had been stored at 4°C in AS-3 for 15 days, 24-hour posttransfusion survival was measured by the double-label procedure with 15 to 20 µCi of ^{51}Cr and 15 to 20 µCi of ^{99m}Tc. The previously frozen RBCs were labeled with ^{51}Cr and the volunteer's RBC volume was measured with ^{99m}Tc fresh autologous RBCs.[8,9]

In six male and eight female volunteers who received autologous transfusion at UMASS, the 24-hour posttransfusion survival of previously frozen RBCs that had been stored at 4°C for 15 days after deglycerolization was measured by the double ^{51}Cr procedure. Two µCi of ^{51}Cr was used to label fresh autologous RBCs for the RBC volume measurement, and 20 µCi of ^{51}Cr was used to label the previously frozen RBCs.[10] The T_{50} reports the length of time that 50 percent of the ^{51}Cr radioactivity present in the RBCs 24 hours after transfusion was detected in the peripheral blood. Blood samples were obtained before, 24 hours after, and 7, 14, 21, and 28 days after transfusion to measure the ^{51}Cr T_{50} value. No correction for ^{51}Cr elution from the RBCs was made in the reporting of the ^{51}Cr T_{50} values. The ITE was calculated from the FTW recovery value multiplied by the 24-hour posttransfusion survival value.[1,11]

Statistical analyses were performed with statistical software (SAS Institute, Cary, NC). A paired *t* test was used to test for significant differences in the results of a randomized study in which 10 volunteers received previously frozen, autologous RBCs stored at 4°C in AS-3 for 15 days after deglycerolization and liquid-preserved, autologous CPD RBCs stored in AS-1 at 4°C for 42 days. A p value of 0.05 was considered significant. Regression was used to describe the posttransfusion survival of the autologous RBCs. Means ± SDs are reported.

RESULTS

A total of 165 units of blood were collected at five sites, frozen with 40-percent (wt/vol) glycerol, and deglycerolized in the ACP 215 instrument. Twenty-three units were not included in the analysis for various reasons. One unit was contaminated; 9 units were kept frozen less than 2 weeks; 2 units were stored in a 4°C refrigerator that had malfunctioned; 3 units leaked during thawing because of improper sealing of the tubing; there was a technical problem with radioactive labeling of 1 unit; in two cases, the full unit was not recovered during processing; 1 unit was stored at 4°C in AS-3 for 20 days instead of 15 days after deglycerolization; three subjects did not present for return transfusion; and one subject had poor venous access and could not receive a transfusion.

In vitro results were obtained for 142 of the 165 units collected. In vivo 24-hour posttransfusion survivals and ^{51}Cr lifespan values were measured in 36 subjects who received previously frozen, autologous RBCs stored at 4°C in AS-3 for 15 days after deglycerolization and in 10 subjects who received liquid-preserved, autologous RBCs stored at 4°C in AS-1 for 42 days.

In vitro measurements in the 142 units showed FT recovery values of 98.6 ± 1.3 percent and FTW recovery values of 87.0 ± 5 percent (Tables 1 and 2). The previously frozen RBCs stored in AS-3 at 4°C for 15 days after deglycerolization showed a mean hemolysis of 0.6 ± 0.2 percent (Tables 1 and 2) in 141 units.

Aerobic and anaerobic cultures showed no bacteriologic growth in 141 of the 142 units of previously frozen RBCs cultured after postwash storage at 4°C in AS-3 solution for 15 days. One in vitro unit grew the skin contaminant *Propionibacterium acnes* on Day 15 and on Day 42. One of the 10 liquid-preserved CPD RBC units that had been stored in AS-1 at 4°C for 42 days also grew *P. acnes* after 3 days in culture (Table 3), although no growth was observed on a prior culture of this unit on Day 39 or on culture 30 days after storage at 22°C. It is our opinion that contamination of the previously frozen RBCs occurred at the time of collection and that contamination of the liquid-preserved RBCs occurred when the culture sample was collected on Day 42.

When the double-label procedure was used to measure the 24-hour posttransfusion survival of previously frozen, autologous RBCs stored in AS-3 for 15 days after deglycerolization and transfused to the 14 volunteers in the study at the NBRL, the mean ± SD value was 75 ± 7 percent; in 13 of these volunteers, the $^{51}Cr\ T_{50}$ value was 29 ± 4 days (Table 4). In 10 of these 14 subjects in whom liquid-preserved, autologous RBCs stored in AS-1 for 42 days at 4°C were transfused, the 24-hour posttrans-fusion survival value measured by the double-label procedure was 72 ± 5 percent, and the $^{51}Cr\ T_{50}$ value was 27 ± 3 days (Tables 4 and 5). Five of the 10 units of previously frozen RBCs stored at 4°C for 15 days had 24-hour posttransfusion survival values of <75 percent, while 8 of the 10 units stored for 42 days at 4°C in AS-1 had 24-hour posttransfusion survival values of <75 percent (Table 5).

At WRAIR, where deglycerolized autologous RBCs stored at 4°C in AS-3 solution for 15 days were returned to their eight volunteers, ^{51}Cr was used to label the deglycerolized RBCs, and fresh autologous RBCs were labeled with ^{99m}Tc to measure the RBC volume. The mean ± SD 24-hour posttranfusion survival value was 78 ± 9 percent, and, for seven of the eight subjects, the $^{51}Cr\ T_{50}$ value was 24 ± 5 days.

At UMASS, the double ^{51}Cr procedure was used for measurements in 14 autologous transfusions of previously frozen RBCs that had been stored in AS-3 for 15 days after deglycerolization. The mean ± SD 24-hour posttransfusion survival value was 78 ± 10 percent, and the $^{51}Cr\ T_{50}$ value was 29 ± 5 days (Table 4).

In the 36 autologous transfusions of previously frozen RBCs performed at three of the study sites, the mean ± SD ITE was 68 ± 8 percent. At the one site where 10 autologous transfusions of liquid-preserved CPD RBCs stored in AS-1 at 4°C for 42 days were performed, the Mean ± SD ITE was 72 ± 5 percent.

In Nageotte WBC counts measured both on the day of deglycerolization and

TABLE 1. RBCs processed in the ACP 215 for in vitro analyses: combined data from five test sites

		Postwash storage at 4°C		
	After thaw	Day 0	Day 1	Day 15
FT Recovery (%)				
Mean	98.7	—	—	—
SD	1.1	—	—	—
N	106	—	—	—
FTW Recovery (%)				
Mean	—	86.8	—	—
SD	—	5	—	—
N	—	106	—	—
Hct (%)				
Mean	61	53	53	51
SD	5	3	3	3
N	106	106	106	106
Hb (g/dL)				
Mean	17.6	14.4	14.5	14.4
SD	1.6	1.1	1.1	1.2
N	106	106	106	106
RBC count (× 10^9/mL)				
Mean	5.9	4.8	4.8	4.8
SD	0.6	0.4	0.4	0.4
N	105	106	106	106
Total WBCs (× 10^6/unit)				
Mean	540	—	9.7	9.4
SD	210	—	7.9	7.5
N	99	—	64	64
Total platelet count (× 10^8/unit)				
Mean	175.8	5.6	1.8	1.8
SD	161.8	21.4	2.5	2.1
N	94	106	105	89
Blood pH at 22°C				
Mean	6.9	6.4	6.4	6.3
SD	0.1	0.1	0.1	0.1
N	75	96	100	106
Supernatant osmolality (mOsm/kg H_2O)				
Mean	4579	296	297	300
SD	295	4	5	4
N	106	106	106	106
Supernatant K+ (mEq/L)				
Mean	14.3	1.3	6.9	24.5
SD	4.0	0.9	3.0	4.9
N	94	106	106	106
Supernatant Hb (mg/dL)				
Mean	607	40	91	174
SD	495	13	31	51
N	105	106	106	106
Percentage of hemolysis				
Mean	—	0.1	0.3	0.6
SD	—	0	0.1	0.2
N	—	106	106	106

TABLE 2. RBCs processed in the ACP 215 for in vivo analyses: combined data from three test sites

	After	Postwash storage at 4°C	
		Day 0	Day 15
FT Recovery (%)			
Mean	98.1	—	—
SD	1.8	—	—
N	36	—	—
FTW recovery (%)			
Mean	—	87.9	—
SD	—	3.6	—
N	—	36	—
Hct (%)			
Mean	60	50	48
SD	7	4	4
N	36	36	35
Hb (g/dL)			
Mean	17.8	13.6	13.4
SD	2.2	1.4	1.3
N	36	36	35
RBC count ($\times 10^9$/mL)			
Mean	6.1	4.6	4.5
SD	0.6	0.4	0.4
N	36	36	34
Total WBCs ($\times 10^6$/unit)			
Mean	513	—	3.7
SD	261	—	3.8
N	36	—	9
Total platelet count ($\times 10^8$/unit)			
Mean	130.4	3.8	2.9
SD	8.1	8.8	4.7
N	35	35	29
Blood pH at 22°C			
Mean	6.9	6.4	6.3
SD	0.1	0.1	0.1
N	27	33	33
Supernatant osmolality (mOsm/kg H_2O)			
Mean	4662	299	302
SD	267	6	5
N	36	36	33
Supernatant K+ (mEq/L)			
Mean	16.3	1.1	22.8
SD	4.5	0.7	5.7
N	36	36	35
Supernatant Hb (mg/dL)			
Mean	883	41	143
SD	793	24	61
N	36	36	35
Percentage of hemolysis			
Mean	—	0.2	0.5
SD	—	0.1	0.2
N	—	36	35

after storage at 4°C in AS-3 solution for 15 days, the total number of WBCs was 1×10^7 per unit, with a range from 1×10^6 to 4×10^7 for 73 units (Tables 1 and 2).

DISCUSSION

The ACP 215 is an automated, functionally closed instrument for the glycerolization and deglycerolization of human RBCs. This instrument uses a disposable 275-mL blow-molded bowl with a diverter, an external rotating seal, and a capacity to process 180 mL of RBCs. If the volume exceeds 180 mL, the RBCs will spill over into the waste solution during the deglycerolization procedure. A mean ± SD in vitro recovery of 87 ± 5 percent of the RBCs was observed in this study; this value is similar to that previously reported for studies in which another instrument (115, Haemonetics) was used.[1,11] Previously frozen RBCs stored at 4°C in AS-3 solution for 15 days after deglycerolization were shown to have a mean ± SD 24-hour posttransfusion survival value of 77 ± 9 percent and 0.6 ± 0.2 percent hemolysis. In a study of human RBCs deglycerolized in the 115 instrument, Moore et al.[12] concentrated the deglycerolized RBCs resuspended in a sodium chloride-glucose solution to remove the supernatant solution on the day of washing and then diluted the RBC concentrates with a Hct of 80 percent with 100 mL of AS-3 before storage at 4°C for 21 days. These authors reported that, after 21 days of postwash storage at 4°C, hemolysis was 0.57 percent and the mean 24-hour posttransfusion survival value was 77 percent when measured by the single-isotope method.[12]

TABLE 3. CPD RBCs stored in AS-1 at 4°C for 42 days at NBRL

	Day 42
Hct (%)	
Mean	53
SD	2
N	10
Hb (g/dL)	
Mean	17.0
SD	0.9
N	10
RBC count ($\times 10^9$/mL)	
Mean	5.7
SD	0.2
N	10
Total WBCs ($\times 10^9$/per unit)	
Mean	1.1
SD	0.5
N	10
Total platelet count ($\times 10^8$/unit)	
Mean	180.1
SD	106.2
N	10
Blood pH at 22°C	
Mean	6.5
SD	0.1
N	9
Supernatant Hb (mg/dL)	
Mean	203
SD	67
N	8
Percentage of hemolysis	
Mean	0.6
SD	0.2
N	8

In our study, the ITE for the deglycerolized RBCs processed in the ACP 215 and stored at 4°C in AS-3 for 15 days was 68 percent. When deglycerolized RBCs processed in the 115 were stored at 4°C in sodium chloride-glucose solution for only 24 hours, the ITE ranged from 75 to 80 percent.[1,11] The reason for the difference between the reduced ITE for

TABLE 4. 24-hour posttranfsusion survival, lifespan, and ITE of RBCs deglycerolized in the ACP 215 and stored at 4°C in AS-3 for 15 days

Test site	Label method		24-hour posttransfusion survival value (Double-label procedure)*	Lifespan (T_{50}, days)	ITE* (%)
Combined		Mean	77	28	68
		SD	9	4	8
		N	36	34	36
NBRL	^{51}CR/^{125}I Albumin	Mean	75	29	65
		SD	7	4	6
		N	14	13	14
WRAIR	^{51}CR/^{99m}TC	Mean	78	24	68
		SD	9	5	8
		N	8	7	8
UMASS	Double ^{51}CR	Mean	78	29	71
		SD	10	5	10
		N	14	14	14
NBRL†		Mean	72	27	72
		SD	5	3	5
		N	10	10	10

* Product of in vitro FTW recovery and 24-hour posttransfusion survival value.
† Control: CPD RBCs stored in AS-1 at 4°C for 42 days.

TABLE 5. Comparison of 24-hour posttransfusion survival using the ^{51}CR/^{125}I albumin double-label procedure in autologous RBCs processed in the ACP 215 and stored at 4°C in AS-3 for 15 days after deglycerolization and autologous CPD RBCs stored in AS-1 at 4°C for 42 days in the same donor at NBRL*

	24-hour posttransfusion survival (%)	
Donor	ACP 215	CPD RBCs in AS-1
1	81	68
2	70	71
3	72	70
4	79	73
5	73	68
6	83	78
7	84	72
8	68	67
9	79	74
10	66	82
Mean	76	72
SD	6	5
N	10	10

* Paired *t* test, 1.3; p = NS.

these two groups of deglycerolized human RBCs is the longer postwash storage period of 15 days for those RBCs processed in the ACP 215.

Ten units of liquid-preserved CPD RBCs stored in AS-1 at 4°C for 42 days exhibited a mean ± SD 24-hour posttransfusion survival value of 72 ± 5 percent when measured by the ^{51}Cr/^{125}I albumin double-label procedure, a finding similar to that observed in a study at NBRL in 1988[7] and not significantly lower than the value of 77 ± 9 percent observed in the present study for the 36 autologous transfusions of deglycerolized RBCs stored at 4°C in AS-3 for 15 days and measured by the ^{51}Cr/^{125}I albumin, ^{51}Cr/^{99m}Tc, and double ^{51}Cr procedures. The ITE of 68 percent for deglycerolized RBCs processed in the ACP 215 instrument and stored at 4°C in AS-3 for 15 days was comparable to that of 72 percent for liquid-preserved CPD RBCs stored at 4°C in AS-1 for 42 days.

One in vitro unit of deglycerolized RBCs grew *P. acnes* after storage at 4°C in AS-3 for 15 days. One CPD unit from a separate donor, which was stored in the liquid state in AS-1 at 4°C for 42 days, tested positive for *P. acnes* on Day 42 but showed no growth in a sample tested on Day 39 or after storage at 22°C for 30 days. These findings suggest that the skin contaminant seen in the deglycerolized RBCs most likely was introduced at the time of blood collection and that contamination of the liquid-preserved RBC occurred during sample collection on Day 42. Information from the ongoing bacterial contamination study of blood by the AABB, ARC, CDC, and Department of Defense (BaCon Study) indicates that this bacterium has not been associated with posttransfusion sepsis.

Concern about the transmission of disease from allogeneic RBCs has led to a call for the quarantine of donor RBCs and subsequent retesting of the donor. Frozen RBCs can be stored for at least 10 years. If the blood donor continues to test negative for infectious disease markers over a 6-month period, the frozen RBCs can be considered safe for allogeneic transfusion. The ability to store the deglycerolized RBCs at 4°C for at least 2 weeks makes this practice even more valuable.

In previous studies, the processes of glycerolizing and deglycerolizing RBCs in the presence and absence of freezing was shown to reduce the total number of WBCs in the deglycerolized unit.[13-17] In the study reported here, in which measurements were made by using the Nageotte chamber with Turk's solution in 73 units on the day of deglycerolization and after postwash storage at 4°C in AS-3 for 15 days, it was shown that the FTW process reduced the total number of WBCs to a mean of 1×10^7 per unit, with a range from 1×10^6 to 4×10^7.

Washing previously frozen RBC to remove the cryoprotectant glycerol reduces not only the number of WBCs in the unit but also the number of biologically active substances that some investigators believe may contribute to the immunomodulation effects in recipients.[18-20]

Several factors affecting the safety and therapeutic effectiveness of an RBC transfusion include the quantity of RBCs in the unit; the survival and function of RBCs, the residual hemolysis and sterility; the residual number and function of WBCs; and the number of residual biologically active plasma and nonplasma substances.

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Appendix O - (The effect of two additive solutions on the postthaw storage of RBCs)

The effect of two additive solutions on the postthaw storage of RBCs

John R. Hess, Heather R. Hill, Cindy K. Oliver, Lloyd E. Lippert, and Tibor J. Greenwalt

BACKGROUND: Sterile systems for freezing and for washing thawed blood will allow the storage of RBCs for more than 24 hours after removal of the cryoprotectant glycerol. This study assessed the effect of two ASs in maintaining deglycerolized RBCs.

STUDY DESIGN AND METHODS: Twenty-four RBC units were stored for 6 days, pooled in groups of 4, realiquoted, sterilely glycerolized, and frozen. One month later, the units were thawed, sterilely deglycerolized by using an automated system (H215; Haemonetics), and stored for 5 weeks in either 100 or 200 mL of AS-3 or an experimental AS (EAS-61). Sterile samples were taken weekly for chemical and morphometric analysis.

RESULTS: The glycerolization and deglycerolization process produced highly comparable RBC units, but it caused a marked reduction of RBC pH, to about 6.4 at the beginning of storage. The addition of acidic AS-3 further reduced the pH, which in turn reduced glucose consumption, lactate formation, and RBC ATP concentrations. Alkaline EAS-61 increased these measures. Hypotonic EAS-61 caused increased cell swelling and hemolysis, despite better RBC morphology.

CONCLUSIONS: Automation of sterile glycerolization and deglycerolization with the H215 works well, but the solutions should be reformulated for extended postthaw storage. This would best be accomplished by raising the pH of the wash solutions by the addition of disodium phosphate or sodium bicarbonate or both, by using alkaline ASs, and by matching the osmolality of the wash solution and ASs.

Frozen RBC storage has been practiced for 50 years.[1] However, it is not commonly used, because it is labor-intensive and is performed in open systems with the potential for bacterial contamination, and because unit quality is difficult to control. The potential for bacterial contamination with the washing of RBCs in open centrifugal systems led the FDA to direct that deglycerolized RBCs be administered within 24 hours of thawing or be destroyed.[2] These shortcomings led to the design and development of closed, automated, and performance-monitored systems for RBC glycerolization and deglycerolization. The system used in this study (H215, Haemonetics Corp., Braintree, MA) is the first such system available for evaluation.

A practical consideration in the design of such a system is the composition of the postthaw RBC storage solutions. Several investigators have demonstrated 2-week postthaw storage of deglycerolized RBCs, and several groups of researchers[3-7] showed that RBCs frozen on the day of collection could be stored for 3 weeks after deglycerolization in AS-3 (Nutricel, Pall Corp., Covina, CA). However, RBCs are rarely frozen on the day of collection. They are usually frozen only after the return of virus testing results or even longer, out to the FDA limit of 6 days.

ABBREVIATION: EAS-61 = an experimental AS.

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To explore the potential for longer postthaw storage at 1 to 6°C, we compared the chemical and morphologic characteristics of RBCs stored in liquid form for 6 days; then frozen, stored 1 month, thawed, and deglycerolized; and finally stored in liquid form for 5 weeks in either 100 or 200 mL of AS-3 or an experimental AS (EAS-61). EAS-61 was chosen as an alternative AS, because it allows the storage of RBCs for 9 weeks at 1 to 6°C.[8] The results of this study show that the deglycerolization process leads to a marked reduction in storage pH at the beginning of postthaw liquid storage. This reduction in pH and subsequent suppression of glycolysis was worse with the use of acidic AS-3. On the other hand, the use of hypotonic EAS-61 led to increased hemolysis. On the whole, the study suggests that better wash and storage solutions can be formulated.

MATERIALS AND METHODS

Volunteers

Twenty-four volunteers donated full units of blood under a protocol approved by the institutional review board of the Walter Reed Army Institute of Research. The donors met standard blood donor criteria.

Storage solutions

The compositions of AS-3 and EAS-61 are compared in Table 1. The significant differences in the solutions are the absence of citrate, the reduced salt content, and the presence of mannitol and disodium phosphate in the EAS-61. The presence of disodium phosphate raises the pH of the EAS-61 to about 8.4 at room temperature, while that of AS-3 is 5.8.

The EAS-61 was made in the laboratory from USP adenine, sugars, and salts and was sterilely filtered into 1-L storage bags. The bags were held at 37°C for 2 weeks. The solutions were then cultured and the cultures incubated for another 2 weeks. When sterility was confirmed by the absence of bacteria growth for 7 to 14 days, the solutions were aliquoted by weight into 150- or 300-mL bags. All connections were made with a sterile connecting device (SCD 312, Terumo Medical Corp., Elkton, MD).

TABLE 1. Composition of the additive solutions used*

	AS-3	EAS-61
Volume (mL)	100, 200	100, 200
NaCl	70	26
NaH_2PO_4	23	0
Na_2HPO_4	0	12
Adenine	2	2
Dextrose	55	110
Mannitol	0	50
Citric acid	2	0
Na_3 citrate	20	0
pH	5.8	8.4

* All concentrations are given in mmol per L.

Study design

We conducted a "pooling" study to evaluate RBC metabolism and physiology over the course of storage. Pooling restricts the largest source of variability in conventional blood storage studies, the differences between the RBCs from different donors, by placing some of the cells from every donor in every arm of the study while maintaining conventional unit size and geometry. In this study, the RBC units were grouped into sets of 4 ABO-matched units. Each set was then pooled, mixed, and realiquoted into identical pooled units.

Specifically, 24 units of whole blood were drawn into 800-mL CPDA-1 bags (Code 4R3420, Baxter Healthcare Corp., Deerfield, IL) and stored for 6 days at 1 to 6°C. On the sixth day, they were pooled in groups of 4, realiquoted, sterilely glycerolized by the automated procedure on the H215, and frozen at –80°C. A month later, the units were thawed, sterilely deglycerolized by the automated procedure on the H215, and stored in 100 or 200 mL of AS-3 or EAS-61. The automated procedure for deglycerolization is a modification of the standard US military procedure of Valeri.[9-11] It uses 12-percent saline for initial cell shrinkage to facilitate glycerol removal and then five washes in 0.9-percent saline and 0.2-percent glucose, and it ends with a final wash in the AS. When 200 mL of AS was used, the second 100 mL was added to the product by sterile tubing connection. Units were then sampled, placed in a 1 to 6°C refrigerator, and sampled weekly for 5 weeks.

In vitro measurements

Samples from stored units were collected, after gentle mixing by inversion, by a sterile sampling procedure in a biologic safety cabinet. A battery of in vitro tests was performed on all units at the beginning of storage and weekly thereafter.

The total Hb concentration, WBC counts, and RBC counts were measured with a clinical hematology analyzer (Hematology Cell Counter System Series 9000, Baker, Allentown, PA). Supernatant Hb was measured spectrophotometrically by the modified Drabkins assay.[12] The percentage of hemolysis was determined by the ratio of free Hb to total Hb. The results are expressed as percentage of hemolysis to compensate for the differences in Hct and Hb concentrations in samples. Centrifuged microhematocrits (Clay Adams, Becton Dickinson, Rutherford, NJ) were performed to avoid the readjustment of cell volume and subsequent errors caused by the isotonic diluent used in the blood analyzers. The MCV was calculated from the machine RBC concentrations and the microhematocrit.

RBC ATP concentrations were measured in deproteinized supernatants. Whole-blood or packed cell aliquots were mixed with cold 10-percent trichloroacetic acid to precipitate blood proteins and then centrifuged at 2700 × *g* for 10 minutes, and the protein-free supernatant was frozen at –80°C until testing. ATP was assayed enzymatically by using a commercially available test kit (Procedure 366-UV, Sigma Diagnostics, St. Louis, MO).

Blood gases and pH were measured on a blood gas analyzer (Corning 855, Ithaca, NY). Thus, pH was measured at 37°C. Phosphate and glucose were measured on a programmable chemical analyzer (Hitachi 902, Boehringer Mannheim, Indianapolis, IN).

The average degree of RBC shape change from discocyte to echinocyte to spherocyte was graded by the RBC morphology index on samples of 400 cells, determined according to the method of Usry et al.[13]

Statistical analysis

Comparisons of means of measured values at given times within the individual crossover trials were evaluated with ANOVA using statistical software (Numerical Algorithms Group Statistical Inserts for Microsoft Excel, NAG, Downers Grove, IL; Microsoft Corp., Redmond WA). Probabilities <0.05 were considered significant.

RESULTS

The measured pH at the beginning of postthaw storage was low in all groups. The pH of the units stored in 100 mL or 200 mL of AS-3 averaged 6.301 ± 0.010 or 6.213 ± 0.010, respectively, and that of units stored in 100 mL or 200 mL of EAS-61 averaged 6.529 ± 0.005 or 6.578 ± 0.005, respectively (Fig. 1A). These low pH measurements were associated with extremely low concentrations of plasma bicarbonate (<1 mEq/L) in all groups.

RBC ATP concentrations averaged 5.6 ± 0.2 µM per g of Hb in all groups at the beginning of storage but differed thereafter, with the EAS-61-stored cells maintaining higher concentrations (Fig. 1B). By 2 weeks, the AS-3-stored cells averaged RBC ATP concentrations of about 2 µM per g of Hb, while the concentration in the EAS-61-stored cells was 4.2 ± 0.2.

Glucose concentrations were higher in the EAS-61-stored cells (Fig. 1C). Moreover, the rate of decrease in the EAS-61-stored cells was greater. This difference in the rate of glycolysis is confirmed by the greater rate of lactate production in the cells stored in EAS-61 (Fig. 1D).

RBCs had a greater initial MCV (about 118 fL) when stored in EAS-61 than did the cells stored in AS-3 (about 110 fL) (Fig. 2A). RBCs had good morphology in all groups at the beginning of postthaw liquid storage, with an average morphology index of 89 ± 1. The morphology was initially identical but was better maintained in EAS-61 at both storage volumes (Fig. 2B). Nevertheless, hemolysis was higher in the EAS-61-stored cells at all time points, including the initial measurement (Fig. 2C).

DISCUSSION

Freezing RBCs has proven valuable for the storage of units of rare blood types, for the storage of autologous units, and for the supplementation of inventory in certain high-use

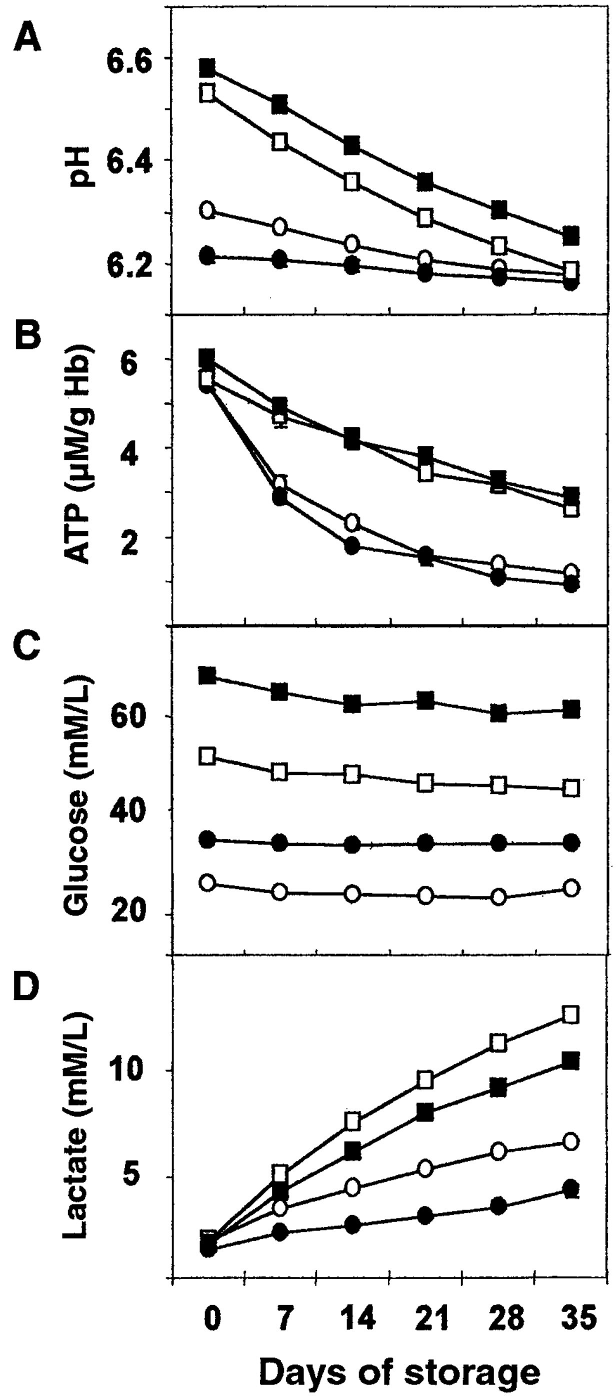

Fig. 1. A comparison of the effects of postthaw storage in two ASs at two volumes on extracellular pH (A), RBC ATP content (B), extracellular glucose concentration (C), and whole-blood lactate content (D). The individual solutions and volumes were: AS-3 at 100 mL (○), AS-3 at 200 mL (●), EAS-61 at 100 mL (□), and EAS-61 at 200 mL (■). The difference in ATP concentrations in RBCs stored in the different solutions at 14 days is about 2 µ*M* per g of Hb or about 120 µ*M* per unit. This is about one-eighth of the ATP produced, as determined from the production of lactate.

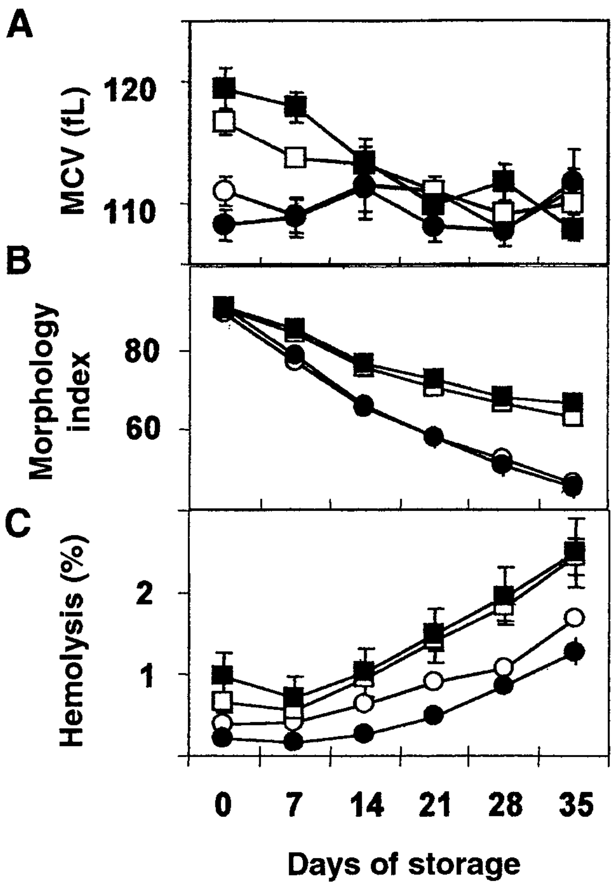

Fig. 2. A comparison of the effects of postthaw storage in two ASs at two volumes on MCV (A), morphology index (B), and hemolysis (C). The individual solutions and volumes were: AS-3 at 100 mL (○), AS-3 at 200 mL (●), EAS-61 at 100 mL (□), and EAS-61 at 200 mL (■). The smaller content of nonpenetrating ions in EAS-61 leads to greater RBC swelling initially, and, despite better preservation of RBC morphology with EAS-61, there was greater hemolysis.

situations where partial automation (IBM 2991, IBM, Armonk, NY) can be used. The technology has proven much less useful in remote locations, where high cost and low levels of training combine to make fully manual systems problematic.

With the development of sterile blood bag connection methods, it became possible to design closed systems for deglycerolizing RBCs. The first such system was built in prototype using a hollow-fiber dialysis cartridge at the former Letterman Army Institute of Research in San Francisco in 1986. The Letterman group went on to demonstrate that liquid storage for as long as 3 weeks after deglycerolization was possible.[6] They then solicited the manufacture of fully automated devices for the sterile processing of frozen RBCs.

The first of those devices is now undergoing preclinical testing. The H215 achieves sterile glycerolization by the addition of the glycerol to the primary blood bag through a sterilely connected 0.22-µm filter. It achieves sterile deglycerolization of RBCs by sterilely connecting the frozen-storage bag to the washing plastic ware, by washing the cells in a special centrifuge bowl with protected rotating seals, and by 0.22-µm filtration of all added solutions. The device is automated, requires approximately 50 minutes to process each unit, and is designed for use with AS-3 as the final wash and storage solution.

We recently developed EAS-61 as a 9-week storage solution and were interested in whether this solution could improve the storage of frozen RBCs as well. As EAS-61 is used in a 200-mL volume with liquid-stored cells, we tested both solutions in 100- and 200-mL volumes.

The pooling design of this investigation appears to have worked well. It produced RBCs with equivalent RBC ATP concentrations, lactate concentrations, and RBC morphology in all four groups at the beginning of the study. It also produced excellent statistical resolution of the differences between the groups that appeared with the addition of the solutions and storage.

The cell-washing process reduced the pH of the washed cells to about 6.4. The addition of acidic AS-3 further reduced the pH to 6.3 or below, which markedly reduced glycolysis. In the absence of significant RBC ATP production, RBC ATP concentrations decreased to values below those associated with successful in vivo RBC recovery at about 2 weeks. Alkaline EAS-61 raised the pH to 6.5 or above and supported two to three times the rate of glycolysis, but it caused increased hemolysis. This increased hemolysis, which was present at the beginning of storage, is probably related to the hypotonic nature of EAS-61, with increased swelling of these fragile cells and the poor match of the tonicity of the EAS-61 and the saline-dextrose wash solution.

The pH of venous blood is normally about 7.35 when measured at body temperature. The addition of the 63 mL of citric acid and monosodium phosphate present in CPD or CPDA-1 lowers the pH of whole blood in a primary collection bag to about 7.2. Further addition of 100 mL of AS-3, which contains 23 mEq per L of monosodium phosphate at pH 5.5, will reduce the pH of AS-3-stored RBCs to 7.0 at the beginning of storage. During the first week of storage, the pH will decline by about 0.2 pH units. These values are above the value of about 6.65 at which rates of RBC ATP synthesis and consumption are balanced. Thus, during conventional liquid storage at 1 to 6°C, RBCs stored in AS-3 show a rise in RBC ATP concentrations during the first 2 weeks of storage and a decline thereafter, as the products of metabolism accumulate in the bag, reduce the pH, and inhibit glycolysis. In this study, ATP consumption exceeded synthesis at all times. This is probably entirely a function of the pH, as adenine, glucose, and phosphate concentrations were adequate.

The data suggest that modification of the cell-washing procedure by the addition of disodium phosphate or sodium bicarbonate to the wash solution to raise storage pH will be useful. We have done this, adding 10 mEq per L of disodium phosphate to the washing solution of a single unit of frozen RBCs, and we were able to raise the initial pH to 6.9. Matching the tonicity of the wash and storage solutions may prevent osmotic RBC lysis, which appears to have occurred when a hypotonic storage solution added after completion of the wash caused marked cell swelling. Otherwise, a high pH to maintain glycolysis and a low final tonicity of approximately 290 mOsm to preserve morphology are probably useful.

Automated frozen blood-processing systems that will allow 2 weeks of postthaw liquid storage will be available in the near future. Improving the wash and storage solutions for even longer postthaw storage should be possible.

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Appendix P - (Buffered washing improves red cell post thaw storage)

•• 347

REPORT ON 12 WEEK STORAGE OF RED BLOOD CELLS IN A LOW CHLORIDE, ALKALINE, HYPOTONIC ADDITIVE SOLUTION

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Objective: To investigate the effect of replacing sodium chloride with an equivalent amount of sodium bicarbonate in an additive solution (AS) with acceptable in vivo results for nine week red blood cell (RBC) storage. To establish how relative volumes of the AS would perform with RBCs derived from 400 to 500mL of CPD blood. Design: RBCs were stored for 12 weeks in an alkaline, hypotonic AS with no chloride. Materials and Methods: Twenty-four leukoreduced CPD RBC units, pooled in ABO & D-compatible groups of three, were mixed and realiquoted into three groups of eight test units. The equivalent of 200 mL/450mL unit of the AS (containing in mM: adenine 2, glucose 110, mannitol 55, disodium phosphate 12, sodium bicarbonate 26, pH 8.51, mOsm 256/Kg H_2O) was added. Adenosine 5'-triphosphate (ATP), hemolysis, mean corpuscular volume (MCV), pH, glucose, lactate, pCO_2, pO_2, supernatant K, Na, Cl, Ca were measured to 12 weeks, 2,3-diphosphoglycerate (DPG) at three weeks. Results: In all three groups the ATP was maintained at >107% of initial concentration for 8 weeks and was >3.15µmol/g Hb at 12 weeks. At 12 weeks, hemolysis was <0.6%. Extra- and intracellular pH started at .7.4 and decreased to .6.5 after 12 weeks. Notably, DPG held at the initial level for one week, was >10µmol/g Hb after two weeks and .6µmols after three weeks. Glucose, lactate, pCO_2, pO_2 and other measurements behaved predictably. MCVs were >121fL at 1-hr and 105fL after 12 weeks in all the mixtures, and >95% of the cells were still discocytes. Conclusion: This low chloride, alkaline, hypotonic additive solution promises to make possible storage of clinically acceptable RBCs for twelve weeks.
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•• 348

FIRST DESCRIPTION OF H VARIANTS IN A GREEK FAMILY – SEROLOGICAL AND MOLECULAR ANALYSIS

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The first H deficient variant, the " Bombay " phenotype, was described in 1952, and was defined by a total lack of ABH activity in erythrocytes and secretions. Its genetic background is due to alterations of the corresponding gene FUT1, which encodes for the L-fucosyltransferase enzyme.

We present the first family with Bombay and para-Bombay phenotype in the Greek population (Caucasians). The determination has been done by use of standard serological methods of ABO and molecular analysis of the FUT1 gene for its genetic alterations.

Serologically we used anti-A, anti-B, anti-AB, anti-D, anti-H tube test and gel test of DIAMED. Conformation tests were performed by the use of ABO known red cells.

We observed anti-A/B/H antibodies (strong) in sera of the members with Bombay phenotype with negative autocontrol (O+, non-secretors). Anti-A and anti-H antibodies (weak) were found in one member of this family with para-Bombay phenotype (B+, secretor) and anti-A, anti-B and anti-H antibodies (weak) in another member with para-Bombay phenotype (O+, secretor).

We isolated genomic DNA from blood from 6 members of the family (DNAzol technique–MRC) and amplified the entire coding region of the FUT1 gene by standard PCR methodology. The amplified fragments (1200 bp) were subsequently fully sequenced by an ABI-310 genetic analyzer. The methodology included 8 primers (forward and reverse). The sequencing revealed two point mutations : 538 C_T/180Gln _stop and 826C_T/276Gln _stop, with an additional silent polymorphism (1088 T_C). The first point mutation and the polymorphism are newly described. This results in a disruption of the transcription of FUT1 gene in the members of the family with Bombay phenotype and a diminished transcription in the members of the family with para-Bombay phenotype.

In conclusion , this is the first Bombay/para-Bombay family case in the Greek population with genetic alterations not previously described. Further investigation will include all members of the family.

•• 349

BUFFERED WASHING IMPROVES RED CELL POST THAW STORAGE

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Objective: Liquid red cell storage is improved when the storage solution pH is increased. This research was undertaken to determine if buffering the deglycerolization wash solution would improve the storage characteristics of deglycerolized blood. Design/Materials and Methods: Twenty-four units were collected into CP2D, leukoreduced, packed to 75% hematocrit and pooled in sets of four of identical ABO. Pooled cells were divided into four aliquots and 100 mL AS-3 added to each aliquot before 4C storage. On the sixth day of storage, the cells were glycerolized in a closed system using the Haemonetics ACP™ 215, and frozen for at least 24 hours. Thawed cells were deglycerolized, two of each set with the unmodified saline dextrose wash, pH 5.5, and two with disodium phosphate (20mmol/L) buffered saline dextrose wash, pH 8.8. One each of the standard and modified wash pairs was stored in AS-3 and the other in an experimental solution (data not shown). Hemolysis and ATP were measured at beginning of post-deglycerolized storage and weekly for 42 days.

Results:

Saline Dextrose	Percent	Hemolysis					ATP % Initial
Solution	Day 7	Day14	Day 21	Day 28	Day 35	Day 42	Day 42
Unbuffered (AS-3)	0.2	0.3	0.5	0.6	0.8	1.0	27
Buffered (AS-3)	0.2	0.3	0.5	0.6	0.7	0.8	61

Conclusion: Liquid blood stored in AS-3 for 42 days has been previously reported having 0.9% hemolysis and 59% of initial ATP. In this study, hemolysis with AS-3 resuspended cells was comparable to liquid stored AS-3 blood, while buffering the saline/dextrose deglycerolization solution maintained ATP levels better than non-buffered solution. Therefore, hemolysis and ATP

results would indicate potential for storage of deglycerolized cells beyond the current 14-day limit. (Supported by Haemonetics, Inc. and US Army MRMC)

•• 350

FROZEN RED BLOOD CELLS, GLYCEROL CONCENTRATION AND FREEZING METHOD AFFECT HEMOLYSIS DIFFERENTLY.

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Objective: A rigorous comparison of the 19%glycerol/-196°C method (LGM) and the 40%glycerol/-80°C method (HGM) for freezing red blood cells (RBC).
Design: A fully automated procedure on a Haemonetics M215 cell-washer was developed for the LGM and the HGM. In a paired study, the effects of (de)glycerolization and freezing were separately studied. Materials and Methods: Whole blood was filtrated, pooled, split and plasma was removed. Of each pool (n=4), each unit was subjected to one of 5 different treatments: HGM, LGM, non-frozen HGM, non-frozen LGM, or no treatment (control). After treatment the cells were stored at 4°C in SAGM. Results: Hemolysis during deglycerolization of non-frozen cells was higher with 40% glycerol (11±3) compared to 19% glycerol (2.4±0.4%). The freeze/thaw step doubled the hemolysis during washing only of LGM-units. Recovery (48±2 g Hb) was dependent on the bowl volume and not affected by the different treatments. Hemolysis after washing was independent of treatment (0.3±0.1%). However, during post-wash storage in SAGM, hemolysis increased strongly with the freeze/thaw step of both freezing methods. Here, the LGM-units showed the highest hemolysis: 0.6±0.2% to 2.3±0.5% from day1 to day 8 after deglycerolization (vs. 0.3±0.1 to 1.3±0.3% for HGM-units). In a separate study it showed that hemolysis of both LGM and HGM cells is reduced (±60%) when AS3 instead of SAGM is used as a storage medium. Conclusion. The M215 is able to process both HGM and LGM frozen/thawed cells with comparable recoveries. The amount of glycerol used as cryoprotectant has an immediate effect on cell integrity. Post-wash stability of HGM cells during cold storage is higher than that of LGM cells. In combination with AS3, prolonged post-wash storage of both LGM and HGM treated cells is feasible.

•• 351

SCREENING OF A POPULATION OF SICKLE CELL DISEASE PATIENTS FOR RARE RHCE ALLELES ASSOCIATED TO CLINICALLY SIGNIFICANT ANTIBODIES

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Background : Many RH variants have been described in the Black population. They are occasionally associated to clinically significant antibodies making transfusion support difficult, specially in sickle cell disease (SCD) patients. Then, only similar variant or deleted-RH red blood cells can be used. We have evaluated the incidence of rare RHCE alleles in a population of SCD patients treated in France.
Patients and Methods : 146 SCD patients from West Indies, West and Central Africa were studied. PCR assays were designed to detect mutations carried by the RHCE alleles and RHD-CE haplotypes known to be associated with antibody production : ceEK, ceAR, ceBI (RH :-18,-19 making anti-RH18) ; (C)ces haplotype (RH :-34 making anti-RH34); ces(340) and ceMO alleles producing a partial e antigen and associated to the production of anti-e.
Results : Allele frequencies were as follows : ceEK and ceAR alleles: 4% ; (C)ces haplotype : 7,5% ; ceMO allele : 2% ; ces(340) : 0,7%. All patients were heterozygous except one who was homozygous for (C)ces. This patient transfused only 3 times produced anti-RH34. These results allow us to estimate the incidence of the phenotypes associated to the homozygous state of these alleles and haplotypes : RH :-18: 1/600 ; RH :-34: 1/190 ; partial e with ces(340) : 1/21000 ; partial e with ceMO : 1/2370.
Conclusion : When an antibody is produced by patients carrying rare RH alleles, no compatible blood can be found. Then, comparing the number of SCD patients treated in France (almost 4000) with the estimation of the rare RH phenotypes in this population, we think that it is relevant to detect these rare phenotypes in SCD patients and in Black donors.
Accordingly, a procedure based on the serological and molecular data to characterize these rare variants is proposed.

•• 352

CHARACTERIZATION AND CONSEQUENCES OF NOVEL *O* ALLELES IN THE ABO BLOOD GROUP SYSTEM

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Objective: This study was conducted to identify hitherto unknown *O* alleles and to evaluate their impact on *ABO* genotyping and our understanding of phenotype/genotype correlation.
Design: Samples referred to our laboratory due to ABO discrepancies and a cohort of group O donor samples were analysed to identify anomalous alleles.
Material and Methods: Blood samples were investigated by serological methods in combination with *ABO* genotyping and DNA sequencing of the *ABO* gene and its regulatory elements.
Results: Analysis of donor and patient samples heterozygous for a common *O* allele and the infrequent O^2 or O^3 alleles showed that different ABO phenotypes (O or suspected A subgroup) could result. In addition, heterozygosity for novel *O* alleles without the 261delG mutation was found in samples with ABO discrepancies (weak A expression and/or weak-absent anti-A in plasma): Two were A^1-like alleles and had nonsense mutations in exon 2 or 6 causing truncation of the glycosyltransferase (R56X or Q108X). In one family a new O^2-like allele with missense mutations 649C>T+689G>A was detected. Whilst the father (O^1O^2) was regular blood group O, the son ($O^{1v}O^2$) had a suspected A subgroup. Screening of other O^2 alleles showed ~25% to have 649C>T. Another group O sample was difficult to genotype and found homozygous for O^1 alleles with 768C>A. Screening among

Appendix Q - (Prestorage leukoreduction may reduce hemolysis of frozen/thawed red cells post deglycerolization)

Title: Prestorage Leukoreduction May Reduce Hemolysis Of Frozen/Thawed Red Cells Post Deglycerolization LE Lippert,[1] MJ Lopatka,[1] HR Hill,[1] JM Salata,[1] JA Holmberg,[2]
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Background: The deglycerolization wash process has been reported to reduce the white cell load in a unit of blood, though not to a level that meets leukoreduction standards. Data from this research compares hemolysis results at 14 days after deglycerolization of non-leukoreduced units to those leukoreduced by filtration performed within 8 hours of collection and subsequently frozen. **Methods:** Twenty-four units were collected into CP2D, leukoreduced (LR), packed to 75% hematocrit and pooled in sets of four of identical ABO. Pooled cells were divided into four aliquots, 100 mL AS-3 added to each, stored at 4C for 6 days, glycerolized in a closed system (Haemonetics ACP™215), and frozen for at least 24 hours. Thawed cells were deglycerolized, two of each set with the unmodified saline dextrose wash, pH 5.5, and two with wash solution buffered with 20 mmoL /L of disodium phosphate, pH 8.8. After deglycerolization, one each of the standard and modified wash pairs was stored in AS-3 and the other in an experimental solution, EAS-64. Hemolysis was measured at the beginning of post-deglycerolized storage and weekly thereafter. The hemolysis results from the cells stored in AS-3 post deglycerolization were compared to units individually collected into CPDA-1 without LR or pooling and processed in the same manner as those units deglycerolized with the standard, unbuffered wash. **Results:** Hemolysis of LR AS-3 cells deglycerolized with the standard wash was 0.2, 0.3, 0.5, 0.6, 0.8 and 1.0% after 7, 14, 21, 28, 35 and 42 days storage, respectively; hemolysis of cells processed in the buffered wash was 0.2, 0.3, 0.6, 0.6, 0.7 and 0.8% at the same time points. **Discussion:**

			Hemolysis (%)		
Group	LR	n=	**Day 14**	Day 42	Day 56
Frozen/Thawed, AS-3, Std Wash	Yes	6	**0.3**	1.0	-
Frozen/Thawed, AS-3, Buffered Wash	Yes	6	**0.3**	0.8	-
Frozen/Thawed, CPDA-1, Std Wash[1]	No	106	**0.6**	-	-
Liquid stored AS-3[2]	Yes	13	-	-	0.20
Liquid stored AS-3[2]	No	13	-	0.51	-

[1] Valeri CR et.al., Transfusion 2001,41:933-939. [2] Babcock, JG et.al., Transfusion 2000,40:994-999.

Hemolysis of deglycerolized cells, also stored in AS-3 but collected in CPDA-1 without LR, averaged 0.6% (n=106) after 14 days storage, double the hemolysis when the blood is LR prestorage. It cannot be determined if the different anticoagulant, CP2D/AS-3 versus CPDA-1, contributed to hemolysis reduction. A similar doubling of hemolysis is observed when AS-3 cells, without LR, are not frozen. **Conclusion:** Hemolysis is reduced when the blood is leukoreduced prestorage and stored in AS-3 post deglycerolization. Phosphate buffering of the wash solution does not appear to alter the outcome.

Appendix R - (psoralen photochemical inactivation of *orientia tsutsugamushi* in platelet concentrates)

Psoralen photochemical inactivation of *Orientia tsutsugamushi* in platelet concentrates

Kevin J. Belanger, Daryl J. Kelly, Frank C. Mettille, Carl V. Hanson, and Lloyd E. Lippert

BACKGROUND: The risk of transfusion transmission of disease has been reduced by the combination of predonation questions and improved transfusion-transmitted disease assays, but the risk is still present. This study was conducted to determine if psoralen photochemistry could inactivate an obligate intracellular bacterium, with documented potential for transfusion, in PCs to further improve safety.

STUDY DESIGN AND METHODS: PCs were inoculated with MNCs infected with *Orientia tsutsugamushi*. The concentrates were treated with amounts ranging from 0.86 to 138 μmol per L of 4′-(aminomethyl)-4,5′,8-trimethylpsoralen hydrochloride (AMT) combined with a constant long-wave UVA light (320-400 nm) exposure of 5 J per cm^2. The effects of photochemical treatment were analyzed by using a mouse infectivity assay along with in vitro testing by PCR, indirect fluorescence antibody, direct fluorescence antibody, and Giemsa staining.

RESULTS: AMT, at 0.86 μmol per L or more, combined with UVA light of 5 J per cm^2, inactivated *O. tsutsugamushi* that contaminated PCs. The PCs that did not receive the combined treatment caused infection.

CONCLUSIONS: The psoralen AMT, in conjunction with UVA light exposure, effectively abolished the infectivity of PCs deliberately contaminated with the scrub typhus organism *O. tsutsugamushi*, as tested in a mouse infectivity assay.

The potential for infectious agents to survive in donated blood components is a continuing concern. To reduce this risk, donors are routinely questioned for exposure history and are screened for evidence of infection with HIV, HTLV, HBV, HCV, and the syphilis spirochete. The recent FDA-ordered recall of approximately 700 units of blood components from soldiers deployed to Fort Chaffee, AR,[1,2] focused attention on arthropod-borne diseases. Because infection can precede overt symptoms, a number of arthropod-borne diseases such as Lyme disease, the newly identified human ehrlichiosis, Rocky Mountain spotted fever, and mite-borne scrub typhus are also sources of potential infection in the blood. Transfusion-transmitted cases of the latter two illnesses have been documented.[3,4] The danger that an asymptomatic donor might give blood during the incubation period,

ABBREVIATIONS: AMT = 4′-(aminomethyl)-4,5′,8-trimethylpsoralen hydrochloride; DFA = direct fluorescence antibody; IC(s) = infected cell(s); IFA = indirect fluorescence antibody; PC(s) platelet concentrate(s); PRP = platelet-rich plasma.

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the window period, was illustrated when several of the donors involved in the recent FDA recall became ill 1 to 3 days after donating,[2] by which time platelet concentrates (PCs) may already have been transfused.

Several approaches have been suggested to make the blood supply safer when predonation questioning and postdonation testing may be ineffective. Among the more appealing approaches is the possibility of mixing a chemical with the blood, as it is drawn or before it is transfused, that would inactivate pathogens in the donated component and render it safe for transfusion, even when the identity of the pathogen may be unknown. Such was the rationale for the use of methylene blue.[5] More recently, the phthalocyanines and psoralens, photochemical compounds that are activated when exposed to visible light and UV light, respectively, have been tested.[6-9] Combination treatment with psoralens and long-wave UV light is under investigation for inactivating pathogens in PCs and has been shown to inactivate several viruses, including cell-associated viruses, cell-free viruses, and selected prokaryotic organisms.[10-13] Its utility against intracellular bacterial organisms is not established.

We tested the effectiveness of the psoralen 4′-(aminomethyl)-4,5′, 8-trimethylpsoralen hydrochloride (AMT) plus UVA exposure for killing the obligate intracellular scrub typhus rickettsiae in infected PCs using an established animal infectivity assay.[14-16] Treatment with 0.86 μmol per L of AMT plus 5 J per cm^2 of UVA light prevents infection in mice.

MATERIALS AND METHODS

Source and preparation of blood components

PC and MNC preparations were derived from whole blood donations. Volunteers, who met AABB and FDA criteria for allogeneic blood donation,[17,18] donated either 64 mL of blood for preparation of MNC isolates or 450 mL of blood for preparation of PCs. Blood donation procedures were performed in accordance with a Walter Reed Army Institute of Research and US Army Surgeon General's Human Subjects Research Review Board-approved protocol.

Isolation of MNCs for rickettsial infection

Sixty-four mL of blood was collected into evacuated tubes containing ACD (L10315-00, Becton Dickinson, Franklin Lakes, NJ) and mixed with an equal volume of sterile 0.9-percent sodium chloride (J5A275, Baxter Fenwal, Deerfield, IL). MNCs were isolated from the diluted blood by density gradient centrifugation, as described by Castleton et al.[19]

Preparation of PCs

A unit of group O D+ blood was collected into CPDA-1 (Baxter Fenwal), and platelet-rich plasma (PRP) was harvested after centrifugation at 2000 × *g* for 3 minutes. PCs were prepared from the PRP by centrifugation at 5000 × *g* for 5 minutes, which removed all but about 50 mL of plasma and resuspended the platelets after allowing the centrifuged platelet bag to remain undisturbed at room temperature for at least 1 hour.

Source of rickettsiae, method of inoculation, and evaluation of rickettsiae in infected cells

The *Orientia tsutsugamushi* used was partially purified by 11 tissue culture passages of Karp strain,[20] in which the rickettsiae were released from the host cells and most eukaryotic material was removed. Infected cells were prepared by incubating partially purified *O. tsutsugamushi* culture material with human MNCs. The rickettsiae infecting the MNCs were evaluated and quantified by examination of direct fluorescence antibody (DFA)-labeled and Giemsa-stained preparations. The details of these procedures are as described by Castleton et al.[19] The number of *O. tsutsugamushi* rickettsiae per infected cell and the percentage of infected cells were determined by microscopic examination of Giemsa-stained preparations.

Preparation of platelet specimens

Samples for evaluation were prepared in 12 × 75-mm polypropylene tubes; they consisted of 1.5 mL of PC, 0.1 mL of infected monocytes, and sufficient AMT dissolved in water to produce a final concentration of 0.86 to 138 μmol per L. In addition, samples containing platelets only or platelets plus infected cells were prepared.

UVA treatment

The contents of a single platelet preparation were divided equally among three wells of a 24-well tissue culture plate and illuminated for 12 minutes in a specially designed illumination device (Model P-09814-86, Cole-Parmer, Vernon Hills, IL; modified with a cooling fan and UVA-transparent sample holder) calibrated to deliver 5 J per cm^2 of UVA light to the 0.5-mL volume of a single well. The contents of the three wells were recombined for subsequent evaluation.

Evaluation of treated platelet inoculum

The recombined, treated platelet preparation (0.5 mL) was injected intraperitoneally into each of a pair of mice. One untreated mouse was caged with the treated mice. All animals used in this study were maintained and handled in accordance with the National Research Council's recommendations for the care and use of laboratory animals.[21] The mice were monitored daily for signs of illness and infection, including ruffled coats and morbidity. When mice displayed symptoms of infection, one mouse of a pair was euthanized for evaluation; mice that remained asymptomatic were euthanized at the end of the 17- to 21-day observation period. A 25-μL sample of blood for assay of *O. tsutsugamushi* DNA by PCR was collected by retro-orbital bleeding. The blood samples were absorbed onto a 2-cm^2

piece of filter paper (#16030, Schliecher and Schuell, Keene, NH), air-dried, and stored at 4°C for later testing. Two impression smears were prepared from the peritoneal exudate, one fixed in absolute methanol at room temperature for 5 minutes for Giemsa staining and the second in cold acetone for 5 minutes for DFA staining.[22,23] Whole blood was collected by cardiac puncture and allowed to clot at 1 to 10°C for 12 to 18 hours; serum was recovered after centrifugation and stored at –20°C for later testing.

Scrub typhus PCR

O. tsutsugamushi genomic DNA was extracted from the blood spots on the filter paper by boiling in 300 µL of 5-percent solution (Chelex-100 resin [Cat No. 143-2832], BioRad Laboratories, Richmond, CA) in sterile distilled water for 10 minutes.[24] The PCR strategy employed first amplifies a 1412-bp region of the *groESL* operon. DNA amplification reagents (GenAmp #N801-055, Perkin Elmer, Norwalk, CT) were employed by using a programmable thermal cycler (Model PTC-100, MJ Research, Watertown, MA). The 47-µL amplification mixtures contained the following (final concentration): 3.5 µL of 10× buffer (1.5 m*M* $MgCl_2$, 50 m*M* KCl, 10 m*M* TRIS [pH 8.3], 0.001% gelatin), 8 µL of dNTP mixture (0.2 m*M* of each dNTP), 5 µL of each primer: 1) #786F 5´-TAC-CAA-CCA-CTG-TAT-GAT-CG-3´ and 2) #2197R 5´-TCA-CGG-ATC-TGT-TCA-CAA-CG-3´ (0.8 *M* each) (Midland, Midland, TX), and 25.5 µL of filtered, distilled water. PCR employed a hot-start procedure.[25] The reaction mixture was heated to 80°C for 10 minutes, and then 2 µL of DNA extract and 1 unit of *Taq* DNA polymerase were added. Amplification proceeded after the reaction mixture was heated to 94°C for 4 minutes, which was followed by 35 cycles each consisting of 94°C for 1 minute, 50°C for 3 minutes, and 70°C for 2 minutes with amplification ending after 4°C overnight.

The second amplification was performed similarly, except that 5 µL each of two primers internal to the first pair, #1149F 5´-ATT-GTA-CAT-GGY-GAT-CAA-TG-3´ and #1667R 5´-TTC-AAC-AGT-TAT-CAC-TCC-TT-3´ (Cruachem, Sterling, VA), and 1 µL of the primary PCR product in place of the extracted DNA template were used; annealing was at 57°C rather than 50°C. The internal primer sequences were provided by G.A. Dasch (Naval Medical Research Center). Appropriate amplification of the extracted DNA was determined by visual detection of ethidium bromide (Sigma Chemical, St. Louis, MO)-stained bands after restriction enzyme digestion with either *Nla* IV or *Hinf* I as described previously.[18,26,27]

Mouse infectivity assay

MNC preparations containing 7- to 15-percent cells infected with *O. tsutsugamushi* were diluted 1-in-16 with PC. On average, each infected cell contained eight organisms, as determined by Giemsa staining. The identity of the infecting organism was confirmed by indirect fluorescence antibody (IFA). The number of organisms in the PC infected cell (IC) material ranged from 4.5×10^4 to 4.3×10^5. The psoralen was added in concentrations ranging from 0.86 to 138 µmol per L, and the material was exposed to UVA light. Each of the mice was injected intraperitoneally with 0.5 mL of the treated test material. The mice were examined daily for up to 17 days for symptoms of infection, such as lethargy, irregular or labored breathing, and deteriorating coat condition. The blood samples were collected by cardiac puncture and retro-orbital bleeding.

Controls

The bioassay system incorporated five controls. The controls, their purpose, and designation (in parentheses) were as follows. One, mice were injected with PCs only to ascertain the effect of handling and injection (PC). Two, other mice received PCs and ICs only to determine the infectivity of the MNC preparations (PC/IC). Three, other mice were injected with a preparation of PCs and ICs but treated with UVA in the absence of AMT to determine the ability of UVA exposure alone to kill the organism (PC/IC/UVA). Four, additional mice were injected with an unirradiated preparation of PCs plus ICs plus AMT to determine the ability of AMT to render the organism noninfective in the absence of UVA exposure (PC/IC/AMT). And, five, an untreated mouse was included to determine the effect of unknown environmental factors.

RESULTS

Mice receiving incompletely treated control samples (PC/IC, PC/IC/UVA, and PC/IC/AMT) containing 1.9×10^4 to 2.0×10^5 organisms became visibly ill 8 to 17 days later; some died 12 to 17 days after treatment, and all showed evidence of infection with the scrub typhus organism in the peritoneal exudate and blood at necropsy 18 or 21 days after injection. *O. tsutsugamushi* DNA was detected in the PC/IC and PC/IC/AMT mice but not the PC/IC/UVA control mice. All control mice gave evidence of scrub typhus infection by Giemsa and IFA staining of peritoneal exudates and by IFA staining of blood specimens. On the other hand, bioassay mice injected with fully treated preparations (PC/IC/AMT/UVA), with AMT concentrations ranging from 0.86 to 138 µmol per L, exhibited no evidence of infection by all measures used. A composite of results is shown in Table 1.

DISCUSSION

The psoralen AMT, in conjunction with long-wave UVA light exposure, effectively abolished the infectivity of PCs deliberately contaminated with the scrub typhus organism *O. tsutsugamushi* as tested in a mouse infectivity assay. Mice receiving incompletely treated PCs, AMT without UVA illumination, or UVA-illuminated PCs without added AMT

TABLE 1. Results of testing

Treatment	Number	AMT (mol/l)	III*	Giemsa**	DFA*	IFA*	PCR
Control mice							
Untreated	3	0	0/3	0/2	0/2	0/3	NT
PC	3	0	0/3	0/2	0/2	0/3	NT
PC/IC	5	0	5/5(2)†	2/2	2/2	3/3	1/1
PC/IC/UVA	2	0	2/2	1/1	1/1	1/1	0/2
PC/IC/AMT20	2	69	2/2	2/2	2/2	2/2	1/1
PC/IC/AMT40	2	138	2/2	2/2	1/2	2/2	1/1
Test mice							
PC/IC/UVA/AMT40	2	138	0/2	0/2	0/2	0/2	NT
PC/IC/UVA/AMT20	2	69	0/2	0/2	0/2	0/2	NT
PC/IC/UVA/AMT10	2	34	0/2	0/2	0/2	0/2	NT
PC/IC/UVA/AMT5	6	17	0/6	0/4	0/4	0/6	0/2
PC/IC/UVA/AMT2.5	6	8.6	1/6‡	0/4	0/4	0/5	0/2
PC/IC/UVA/AMT2	4	6.9	0/4	0/2	0/2	0/4	0/2
PC/IC/UVA/AMT1.5	4	5.6	0/4	0/2	0/2	0/4	0/2
PC/IC/UVA/AMT1	4	3.4	0/4	0/2	0/2	0/4	0/2
PC/IC/UV/AMT0.5	4	1.7	0/4	0/2	0/2	0/4	0/2
PC/IC/UVA/AMT0.25	4	0.86	0/4	0/2	0/2	0/4	0/2

* Number positive/number tested.
† Death.
‡ Animal became ill and died of causes unrelated to rickettsial infection.

became ill; some died, and all showed evidence of *O. tsutsugamushi* infection in peritoneal exudate and/or blood samples. AMT concentrations as low as 0.86 µmol per L, combined with UVA illumination, were effective; AMT at concentrations more than 100 times greater than 0.86 µmol per L, without UVA exposure or with UVA illumination alone, were ineffective.

In two instances, however, the results of the various tests on samples from mice receiving the same treatment did not corroborate one another. In the first instance, the inability to amplify *Orientia* DNA in samples from mice receiving infected PCs without AMT but exposed to UVA light (PC/IC/UVA) is inconsistent with all other testing, the results of which indicated infection. At least two explanations are offered. Perhaps the UVA exposure damaged the organisms' DNA sufficiently to prevent DNA amplification in these two specimens. Alternatively, the failure to amplify DNA may have resulted from inadequate specimens or technical failure of the PCR assay system. Nonetheless, the preponderance of evidence indicates the mice were infected by the incompletely treated platelet material. In the second instance, the peritoneal exudate of only one of two mice receiving infected PC with the highest dose of AMT, but not exposed to UVA (PC/IC/AMT40), contained cells that were stainable by DFA methods. Here, too, all other testing indicated the animals were infected with scrub typhus.

This research did not directly address the question of toxicity. However, results of other research would indicate that the concentrations of psoralen used in these experiments do not pose a great risk for toxicity. The main concern is toxicity to the potential platelet recipient. Given a typical transfusion of two doses of pooled platelets consisting of 6 PCs each that were treated with 0.86 µmol per L, the total dose of AMT is 0.15 mol (0.86 µmol/L × 15 mL/PC × 12 pooled PCs). Furthermore, only a fraction of total AMT—approximately 12 percent, according to Wagner et al.[28]—remains unbound after UVA illumination. Therefore, 0.018 µmol (12% × 0.15 µmol) circulating homogeneously in the estimated 4835-mL blood volume of a 72.5-kg, 1.73-m person[29] represents an unbound AMT concentration of 3.72×10^{-4} µmol per mL. Even if the amounts of AMT were increased 10- to 30-fold to inactivate more-resistant blood-borne pathogens, the residual unbound AMT would remain well below the lowest dose producing a positive mutagenic response in Ames testing, 4 × $10E^{-2}$ µg per mL, as shown by Wagner et al.[28] Should still higher doses be required, strategies to remove the unbound psoralen, such as quenchers or a removal device, would likely be required.

The second concern with regard to toxicity is damage to platelet function by psoralen and/or UVA irradiation. AMT, at all but the highest concentrations used in these experiments, is not expected to compromise platelet integrity. Margolis-Nunno and coworkers[30] demonstrated full platelet function at 86 µmol per mL with 7 J-per-cm^2 treatment in plasma or at 172 µmol per mL with 38 J-per-cm^2 treatment when oxygen is removed or when compounds that quench photoreactants are included.

A similar psoralen derivative, S-59, has been used in a photochemical treatment process for pathogen inactivation in PC.[31-33] The S-59 process does not require the removal of oxygen or the addition of quenchers to the PC. It includes a device to lower the concentration of residual free S-59 after illumination. In vitro studies and clinical studies have shown that platelet biologic function is largely preserved after the S-59 photochemical treatment process.[34]

Direct treatment of PC with a psoralen such as AMT has the potential of significantly reducing the transmission of blood-borne disease. The effectiveness of psoralen treatment against viral contaminants is well established.[9-12] The process can also be effective against prokaryotic intracellular pathogens, as demonstrated in this study. Furthermore, the transmission of disease organisms, not generally excluded by predonation questioning or postdonation testing, would be minimized. Some measure of protection might also be afforded against disease transmission when blood is collected during the prodromal stages of infection. This seemingly simple and effective blood-sterilization technology deserves wider evaluation.

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Appendix S - (Inactivation of *P. falciparum* by riboflavin and light)

•• P-488

INACTIVATION OF *P. FALCIPARUM* BY RIBOFLAVIN AND LIGHT

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Objective: Avoidance of malaria transfusion transmission depends almost exclusively on excluding donations based on donor's disease history, malaria prophylaxis and travel or residence in endemic areas. A pathogen inactivation method, which inactivates the parasite in collected blood, has the potential to reduce or eliminate transmission because of screening failures and allow collections from donors traveling or residing in endemic areas. Design / Materials and Methods: *Plasmodium falciparum* was added to human red cells resulting in a 6% to 13% parasitemia. Parasitized red cells were diluted to 38% hematocrit with up to 500 microM riboflavin (RF) and exposed to visible light for up to 1 hour. Samples of treated red cells, infected but untreated red cells and partially treated materials (light only or RF only) were cultured in modified RPMI media. Parasite viability was assessed with an ELISA assay for parasitic lactate dehydrogenase. Results: In one set of experiments, parasite viability in samples treated with 500 microM RF and illuminated for 1hour dropped to <half initial by day 4 and to <5% original by day 8. The response was delayed by about 2 days when the illumination time was reduced to 45 min. Parasitized red cells treated with 500 microM RF suppressed parasite growth in absence of illumination 6–8 days after treatment with recovery by day 14–18. Conclusions: Complete suppression of parasitic viability was observed when parasitized red cells were treated with 500 microM RF and 60 minutes of illumination. Parasitized red cells treated with shorter illumination times appear to have delayed and yet complete suppression of parasite viability. Unilluminated controls with RF solution exhibited decreased short-term parasite growth but eventual recovery. (Supported by Gambro BCT and US Army MRMC)

•• P-489

DEVELOPMENT OF AN ANIMAL MODEL TO STUDY LEUKOREDUCTION AND TRANSMISSION OF A PRION AGENT

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Objective: We used a well characterized animal model (Syrian Golden Hamster) to determine the effectiveness of white blood cell (WBC) reduction of hamster blood by the use of standard blood bank collection bags and integrated leukoreduction filters. These studies are a prelude to prion propagation studies using non-leukoreduced and leukoreduced (LR) blood products from hamsters previously infected with the 263 scrapie strain. Design: Given the reported association of prions with WBCs, the titer of prion infectivity should decrease following removal of WBCs. As there is no prior data, we first investigated the efficacy of leukoreduction when hamster blood or its components were processed using standard blood bank packs and integrated leukoreduction filters. Materials and Methods: Preliminary studies were carried out using the Leukotrap RC-PL or WB systems with units of CP2D anticoagulated, pooled blood, recovered by heart puncture, from uninfected (control) hamsters. WBC counts in samples of whole blood, packed RBCs, platelet-rich plasma (PRP), and platelet-poor plasma (PPP) taken before and/or after LR by filtration were assessed by flow cytometry and manual methods. Results: Compared to pre-filtration levels, WBC counts were reduced by approx. 3 logs10 for all blood components using either LR filter system and provided post-filtration WBC levels of 1-10 WBCs/uL. Conclusion: Standard blood bank packs with integrated LR filters provide a suitable means to produce leukoreduced hamster blood components to be used for an animal model of prion propagation.

•• P-490

CLEARANCE OF A BSE-DERIVED AGENT BY PROCESSES USED IN THE MANUFACTURE OF HUMAN IMMUNOGLOBULIN.

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Objective: There is still uncertainty over how the agent of variant Creutzfeldt-Jakob disease (vCJD) would partition during the manufacture of plasma derivatives.
Here we have used a BSE-derived agent as a vCJD model to determine removal of infectivity by selected steps used to manufacture intravenous immunoglobulin (IVIgG).
Design: Three steps used in the SNBTS process for IVIgG were examined both individually and in series (with equivalent steps also being used in albumin production).
Material and Methods: The starting material for each experiment was 'spiked' with microsomal fraction of brain from BSE 301V infected mice and infectivity was determined in resultant fractions using a murine bioassay.
Results: Most 301V infectivity partitioned into Fraction I+III (log reduction 2.1) which is discarded during IgG production. Infectivity remaining in Supernatant I+III was reduced by AP20 glass fibre depth filtration (log reduction 0.6) and then to below the limit of detection by Seitz KS80 depth filtration. Although the different steps operated in series complemented one another, the overall reduction of infectivity was not equal to the sum of the reduction factors measured separately for each step, indicating that it would be inappropriate to add together reduction factors that had been derived for each step in isolation.
Conclusions: It is concluded that vCJD infectivity should be substantially removed from IgG solutions by a combination of Fraction I+III precipitation and Seitz KS80 depth filtration.