RBC Survival Validation in Adult Humans Under Condition of Normal RBC Survival

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RBC SURVIVAL VALIDATION IN ADULT HUMANS UNDER CONDITIONS OF NORMAL RBC SURVIVAL

SUBMITTED BY:

John D. Roback, MD PhD
Associate Professor, Pathology and Laboratory Medicine
Director, Center for Transfusion and Cellular Therapies
Emory University School of Medicine

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SPONSOR:

John D. Roback, MD PhD

Study Summary:

Study phase: Phase I

Duration of study: 1-2 years

Methodology: Blood is collected from healthy subjects, processed into packed red blood cell units, and either immediately afterwards or 40-42 days later the packed red blood cells are labeled with biotin. The biotin-labeled red blood cells are then transfused back to the donor (autologous transfusion), and subsequently blood samples are taken from the subject for up to 154 days (22 weeks) to track survival of the labeled red blood cells.

Study Site: Emory University Hospital

Approximate number of subjects: 8

Investigator's affiliation: Department of Pathology and Laboratory Medicine, Emory University School of Medicine

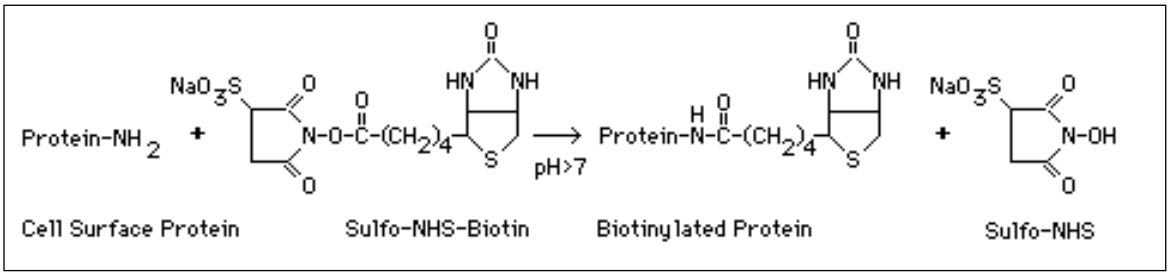
Background Information / Significance:

Name of drug: "biotinylated red blood cells (b-RBCs)"

Active ingredients: Biotin (that is covalently attached to human adult and red blood cells).

Pharmacological class: Water soluble B group vitamin.

Structural formula: We will utilize sulfo-NHS "short chain" biotin (MW=443.43) in a reaction with the amino groups on RBC surface proteins as shown in the following reaction diagram:



Formulation: Indicated as "Biotinylated Protein" in the reaction diagram above.

Dosage: The concentration of sulfo-NHS short chain biotin to be used in each labeling reaction will range from 2-18 μg / mL of packed RBCs. Previous results suggest that these concentrations of biotin will produce RBCs with (on average) 25 – 250 pmol of biotin / mL of packed RBCs. Thus, a study in which 3 aliquots of b-RBCs are infused (25 mL each; labeled with 2, 6 or 18 μg / mL of packed RBCs) will result in the transfusion of approximately 8.8 nmol of biotin (3.8 μg). As a point of reference, this amount is well below the recommended daily intake (RDI) of biotin for adults of 100 μg; a typical multivitamin contains about 30 μg of biotin. Thus, this dosage should be safe.

Route of administration: Intravenous.

Summary of results from prior clinical studies and clinical data to date: There is no known toxicity of biotin in doses vastly exceeding those we propose [1, 2]. Daily doses up to 200 mg orally and up to 20 mg intravenously have been given to newborn infants to treat biotin-responsive inborn errors of metabolism and acquired biotin deficiency. No toxicity at these doses under these conditions has been reported [3-5].

Based on the growing number of published reports using biotinylated RBCs for measuring RBC volume and RBC survival in humans [6-10], the use of biotinylated RBCs is becoming

increasingly accepted as a safe and effective method for determining RBC volume and survival. Biotinylated RBCs have been used to study RBC volume and RBC survival in humans since 1988 [1]. Since that time, there have been no definitive risks of biotinylated RBCs noted in studies in adults in which a total of 96 individuals have been studied [1, 6, 9, 11-16]. In addition to these studies in adults, we are aware of 139 newborn infants in the first months of life who have been studied with biotinylated red cells also for measurement of red cell volume and red cell survival [7, 10, 17, 18]. With the exception of one previous paper [6], none of the other reports described any safety problems with biotinylated RBCs. In the paper by Cordle et al, 15% of the adult subjects developed antibodies to biotinylated RBCs. In none of these adult subjects were antibodies to biotin *per se* identified. Moreover, the antibodies to biotinylated RBCs were transient and did not affect RBC survival, a finding which is supported by a study of biotinylated RBCs administered to adult baboons [19].

Risks and benefits to study subjects: There will be no specific benefits to the 8 subjects to be studied with this protocol.

The risks are those that may be seen with blood collections and blood transfusions. These include: feeling queasy, throwing up, chills or feeling cold, fainting, feeling light in the head, seizures, discomfort around the needle, bruising, infection, swelling around the needle site, blood loss, and red cell destruction. Other risks could include skin rashes and hives. Since only a small amount of blood will be drawn, these risks are probably minimal.

Transfusion recipients may also potentially get a blood infection when they are transfused with the biotinylated red blood cells, although we take a large number of precautions in the lab when we label the RBCs to minimize the chances of this problem, which would be a very rare occurrence.

Many volunteers and patients in other centers have participated in a similar study, and they have not noted any of these adverse effects. The main risk they did observe was the occasional development of an immune response to the biotin label on the RBCs; in the same way that transfusion recipients develop antibodies against alloantigens on transfused RBCs, these recipients can also form antibodies against the novel antigen formed by biotin, the crosslinker, and the crosslinked target on the RBCs [20]. There appear to be no adverse effects of these antibodies by themselves (eg, no effects of recipient biotin levels or function of biotin-dependent processes). Furthermore, the risk of developing these antibodies appears to be reduced when using lower doses of biotin for labeling (eg, $\leq$ 18 μg/mL such as we propose in this protocol). Nonetheless, under the very specific case where study subjects who have developed these antibodies are later transfused with additional biotin-labeled RBCs, the b-RBCs in the second transfusion are eliminated from the recipient's circulation with accelerated kinetics. Because of these occurrences, the FDA requires that recipients be tested for the presence of anti-biotin antibodies following their first transfusion of biotin-labeled RBCs, and that the subset of recipients who make anti-biotin antibodies be eliminated from future studies where biotin-labeled RBCs are transfused.

Description of the population to be studied: Healthy adult volunteers.

Description and justification for the dosing regimen and treatment period: In this study the non-radioactive, multi-biotin-labeling method for labeling RBCs for use in studies of RBC survival will be validated in normal, non-anemic adult volunteers. The dosing regimen and treatment period are based on published studies from Dr. Widness' group at the Unversity of Iowa and their collaborators [9, 14, 20-25] previously developed. Autologous RBCs that are collected, processed into packed RBC units, and then either not stored or stored under refrigerated conditions for 40-42 days, will be labeled with up to 3 separate densities of biotin (2, 6 and 18 μg per mL RBC), and re-infused into the donor. Our hypothesis in this experiment is that we at Emory will be able to recreate the effective methodology for tracking RBC survival pioneered by the Iowa group. Specifically, we will be able to measure RBC survival of up to 3 populations of autologous RBCs in normal adult volunteers using a non-radioactive biotin

labeling methodology. We anticipate that RBC survival of the three biotinylated RBC populations will agree with one another (and with published determinations using the standard ^{51}Cr method). These measures of RBC survival will be conducted in 8 normal adult volunteers studied under normal, non-anemic, steady state conditions during the 154days (22 weeks) following transfusion. Following completion of these studies, and after validating the methodology, we will be in a position to pursue novel studies in which quantification of transfused RBC storage will be an important endpoint.

Compliance: The clinical study will be conducted in compliance with the protocol, SOPs, and the federal, state and local regulations including requirements within the approved FDA IND.

Objectives / Rationale / Research Question: Our research group and collaborators at Emory study diverse aspects of blood banking and transfusion medicine in order to improve the clinical efficacy and safety of RBC transfusion practice. Within this area of investigation, we have a number of ongoing and anticipated studies that would benefit from the use of biotinylated RBCs to track survival of banked RBCs after infusion: (1) We are NIH-funded to identify biomarkers that can predict survival of transfused RBCs. Thus, we would like to compare predictions of RBC survival after transfusion, which we make using these potential biomarkers, with actual RBC survival after transfusion determined by labeling RBCs with biotin, infusing them into recipients, and then collecting post-transfusion samples for flow cytometric quantitation of in vivo RBC survival (this IND application specifically covers these studies). (2) For anemic patients with antibodies against RBCs, in particular either auto-antibodies or broadly reactive allo-antibodies of indeterminate specificity, we would like to develop methods for an “in vivo crossmatch” where small aliquots of donor RBCs that appear to be least-incompatible in vitro (using a standard Coombs crossmatch) are each labeled at different biotin densitites and are then infused simultaneously to determine, in vivo, which donor RBCs survive the longest; the remaining unit from that donor will then be transfused (to be the subject of a future IND application). (3) Addtionally, we would like the ability to study endogenous RBC survival in healthy volunteers and patients (for example, those with sickle cell disease) by drawing a small amount of blood, labeling the RBCs with biotin, and then re-infusing that blood back into the donor to track their endogenous RBC survival (to be the subject of a future IND application).

In order to pursue these and other important clinical transfusion studies we need to first validate the biotin-labeling procedure in human subjects at Emory. This is the goal of the present protocol.

The Research Hypothesis is: We will be able to use biotin-labeling to track RBC survival in volunteers, and the methodology will be validated for later studies by achieving both the Primary and Secondary Objectives (below).

The Primary Objective is: To confirm that we can label RBCs with biotin, at up to 3 different densities, and then distinguish the differentially labeled RBCs from one another both after labeling and subsequently after they have been transfused back to the donor, allowed to circulate, and then re-identified in phlebotomy samples.

The Secondary Objective is: To demonstrate that b-RBCs can be safely transfused back to autologous subjects without any adverse reactions (other than possibly the development of antibodies against biotin-labeled RBCs).

Clinical Study Design:

Primary endpoints: Determination of the length of time biotin-labeled RBCs can be detected

following infusion; determine the length of time that differentially labeled RBCs can be distinguished from one another after transfusion.

Secondary endpoints: Measure the occurrence of adverse effects, including development of anti-biotin-RBC antibodies.

Information needed to answer the research question: Samples of peripheral blood collected at designated times after transfusion of biotinylated RBCs.

Study design: Single-arm study.

Intervention: The intervention is transfusion and subsequent tracking of autologous biotinylated RBCs (b-RBCs; the investigational product). Specifically, the steps include: collection of blood from the subject (up to 500 mL), processing of blood into a packed RBC unit (of the type used for transfusion), storing the unit at 2-6C for 40-42 days (or, in some cases, not storing the unit at all), withdrawing blood from the unit under sterile conditions, sterilely labeling aliquots of this blood with up to 3 pre-defined concentrations of biotin labeling reagent (2, 6, 18 μg / mL of RBCs), re-infusing the b-RBCs back to the recipient, and then taking serial blood samples from the recipient (typically, weekly for up to 154 days) to track the survival of the infused b-RBCs.

Although under ideal circumstances the subject would agree to participate for the maximal study duration of 112 days (from day of blood collection through maximum blood storage period [42 days] and then, after receiving transfusion of b-RBCs on day 42, for another 154 days of weekly blood collections) the subject may elect to discontinue their participation at any time.

There is no randomization for this study. The first 2 donors will not have their blood stored after collection; they will receive transfusion of b-RBCs on the same day blood was collected in order to more rapidly and specifically test the biotin-labeling and post-transfusion detection procedures. The next 6 donors will more closely approximate the protocol for future studies in which blood is collected and stored under standard blood bank conditions prior to biotinylation and transfusion. All confidential information will be stored in locked cabinets.

As described above, the risks are those that may be seen with other studies in which blood collections and blood transfusions are performed. These include: feeling queasy, throwing up, chills or feeling cold, fainting, feeling light in the head, seizures, discomfort around the needle, bruising, infection, swelling around the needle site, blood loss, and red cell destruction. Other risks could include skin rashes and hives. Since only a small amount of blood will be drawn, these risks are probably minimal. Transfusion recipients may also potentially get a blood infection when they are transfused with the biotinylated red blood cells, although we take a large number of precautions in the lab when we label the RBCs to minimize the chances of this problem, which would be a very rare occurrence.

An additional possible risk specific to transfusion of b-RBCs is the occasional development of an immune response to the biotin label on the RBCs. Since the initial studies performed using b-RBCs in the early-1990's, the biotinylation protocol has been optimized; recent evidence suggests that the risks of making these antibodies can be reduced through the use of lower concentrations of biotin during the labeling reaction (eg, $\leq$ 18 μg/mL, as we propose to use in these studies). Should a study participant develop these antibodies, there appear to be no adverse effects of the antibodies by themselves (eg, no effects of recipient biotin levels or function of biotin-dependent processes). Nonetheless, the FDA requires that recipients be tested for the presence of anti-biotin antibodies following their first transfusion of biotin-labeled RBCs, and that the subset of recipients who make anti-biotin antibodies be eliminated from future studies where biotin-labeled RBCs are transfused.

The study subjects will experience no specific benefits during the course of this study. However, we may learn things about blood transfusion by performing these studies that may one day improve the outcomes of patients that require blood transfusion.

Inclusion and Exclusion Criteria of the Subjects:

Inclusion Criteria: Adult subjects of 18-65 years of age will be considered for enrollment; subjects will be enrolled without preference to gender, race or ethnic background. The subjects must be in good health.

Exclusion Criteria: Anemia (Hb < 8 g/dL), chronic diseases (diabetes, heart or lung disease, hypertension [if poorly managed], peripheral vascular disease), ongoing consumption of biotin or raw egg supplements, a history of bleeding disorder or evidence of anemia on initial screening will be excluded. Any subjects who are pregnant or plan to become pregnant will be excluded.

Informed Consent Process: We plan to recruit subjects from our existing registry of transfusion research donors. Most of these donors have previously consented to participate in multiple transfusion research studies, which include studies in which they donate blood and also studies in which they are transfused with blood. Either Dr. Roback or Ms. Shannon Bonds will present the study to potential subjects, describing the procedures (blood draw, transfusion of their autologous blood after it has been labeled with biotin, and subsequent small volume blood draws to track circulation of b-RBCs). Compensation will also be described (up to $40 for the initial donation, $10 for transfusion of b-RBCs, and then $5 for each sample of post-transfusion blood we draw over a total of 154 days post-transfusion). The subjects will be instructed that they can withdraw from the study at any time. The subjects will then have an opportunity to ask any questions, after which they will have the opportunity to review and sign the consent.

Procedures:

Interventions: Collection of up to 500 mL blood; preparation of the b-RBC investigational product (labeled using concentrations of either 2, 6 or 18 μg/mL biotin reagent); IV infusion of up to 75 mL packed RBCs (25 mL of pRBC at each of the 3 labeling densities), and sequential blood draws (< 5 mL each) to track b-RBC survival over the course of up to 154 days.

Medications: the only restriction is the use of biotin or raw egg-containing supplements which by block or otherwise inhibit our ability to accurately quantify b-RBCs. All other medications may be taken during the study.

Compliance: as the investigational product will be administered is by study team, there are not expected to be any compliance issues.

Adverse Events:

Definition of adverse events and anticipated types of adverse events: For the purposes of this study, we define adverse events as those events that the subject encounters (during the blood collection, during the transfusion, or following the transfusion) which cause pain, discomfort, other morbidities, or mortality. Anticipated adverse events during blood collection include: feeling queasy, throwing up, fainting, feeling light in the head, seizures, discomfort around the needle, and bruising. Adverse events that might occur during transfusion include skin rash, hives, fever, chills or feeling cold, infection, swelling around the needle site, and red cell destruction. Possible adverse events that may occur following transfusion include the detection of a new antibody against biotin-labeled RBCs.

Plan for collection of adverse events: Subjects will be monitored closely during the blood collection and transfusion procedures by staff trained to detect these adverse events. If such events occur, they will be entered into the case report forms (CRFs). The detection of antibodies against biotinylated RBCs will be based on testing performed on each sample collected following transfusion; these results will likewise be entered into the CRFs.

Follow-up treatment: Follow-up is dependent on the adverse event, and can range from elevating the legs and applying a cold compress to the back of the neck (fainting and light headedness) to hospitalization (infection or red cell destruction).

Data and Safety Monitoring Plan:

Meeting reporting requirements: All adverse events will be collected on each volunteer. We will report adverse events to the IRB as required by IRB P&P and as part of regular progress reports. In addition, we will submit IND safety reports as required by FDA regulations and report Serious Adverse Events to the FDA as part of the IND progress reports.

Plan for reviewing data for safety: Given that other investigators have performed similar studies using b-RBC infusion with no safety problems, we believe this single-site clinical trial presents only minimal risks. As such, we propose that the PI (Dr. Roback) will monitor data and safety, ensuring participants' safety on a daily basis. Monitoring will occur in real-time (or within 1-2 hours of the occurrence of any adverse events). Thus, initially we will not use an independent Safety Officer (SO) or Data and Safety Monitoring Board (DSMB). Should unexpected risks or problems occur, we will either appoint an SO or DSMB to review study records. In addition, we will arrange for an impartial observer with knowledge of clinical trials to assess the study for data quality and protocol compliance both mid-way through the study (after 4 subjects have completed the study) as well as at the conclusion of the study. This observor will also monitor the study to ensure protocol compliance as well as accuracy and completeness of documentation.

Data Collection, Monitoring and Access:

Overview: All data will be collected, stored and analyzed in consultation with Kirk Easley from Biostatistics. Mr. Easley is a co-investigator on one of the grants supporting this study (National Institutes of Health Program Project Grant, 2 P01 HL 086773-06A1: Mechanisms and interventions addressing serious hazards of transfusion and cellular therapies. 2015 – 2020), where his specific charge is to direct a Biostatistics and Data Management Core.

IT resources: The Information Technology (IT) Department of the Rollins School of Public Health is a state-of-the-art desktop and server infrastructure that supports over 2500 users. From the ground up, it was designed to be modular and expandable so as to give the School the greatest computing capability, flexibility and growth potential. The server environment is based on a combination of UNIX and Windows 8 and can be divided into a number of service areas: The DataFax application runs on a dedicated Unix server that also runs statistical software SAS. Firebird server specification: A Unix Solaris X series server with 8 cores of 2.4 GHZ processors speed, 36 GB RAM Memory, 2 TB RAID 5 disk space, 1 Sun E series tape library tape drive; 2 modems (with one additional modem in reserve) are connected to phone lines with roll over capability. A Linux cluster server with 8 nodes for parallel computing with statistical software (SAS and S-Plus) will be used for analysis in the Roback program project. Cluster specifications: Four R710 , two R720 and one R810 running Linux Operating System; 102 cores , 2.2 GHZ processors, 640 GB total memory, 500 TB disk space on an EMC VNX 5700 storage array and data domain secure backup solution. The servers are housed in the

locked computer room within a secured network operating center (NOC) of the Rollins School of Public Health with 24x7 monitoring, and environmental sensors and climate controls. The servers are accessible by staff within the RSPH building via the school's network which consists of ATM, FDDI and Ethernet hardware running TCP/IP. The staff have PC computers which interface to the server using X-ming. Professional system administrators maintain and operate the servers and are responsible for ensuring performance, uptime and system maintenance, including security and application-layer patching. In addition, their services comply with Emory University Information security practices and controls.

The core of our computer services is provided by high performance computing cluster. Storage is provided through our SAN (storage area network) over a fiber channel network, with 54 terabytes of RAID-protected HIPAA-compliant storage local on the cluster. The server hosts analysis and programming tools including: SAS, SPlus, Fortran 77/90, C, C++, Java, R, MATLAB and IMSL. The application environments are 64 bit and parallel computing enabled when the product supports the capability. Services are provided to the desktop using the X Windows and Sun Grid Engine interfaces. The files systems are GFS2 based for efficiency and stability. Our researchers also have access to a central cluster computer called Ellipse, which has 1024 CPUs and is a high-memory computing environment. We also supply through our Citrix frame 10 open use high end computing desktops. 1,024 CPU environment, high memory nodes.

They also run a number of database services that range from SQLServer 2000-2008 and also an instance of Oracle 10g that are provided across both the VM windows environments and the LInux systems. These databases are used to services a number of application systems across our administrative and research needs. Database accounts are available for any faculty as requested. Web-based access to databases is provided by Adobe ColdFusion application server, which runs on a 440 MHZ Sun Ultra 10 with 512 MG of main system memory and 9 GB of mirrored disk space.

The RSPH network consists of Fast Ethernet hardware running TCP/IP. Gigabit Ethernet provides high-speed transmission to each of 10 floors and across the RSPH campus buildings and all the other buildings across campus. Ethernet provides high speed (100 Mbps) access to most desktop computers and peripheral devices. The network terminates at over 2500 locations. The RSPH network is connected to the Emory Campus backbone via a 1gigabyte Ethernet connection, making campus services and wide area network services readily available. We also have an extensive wireless network providing "N" class connections and speeds that cover all of our buildings and the nearby external areas. This network has guest services as well as secure services for our faculty and staff. Our voice communications are connected into a Unified Communications system that provides phones through VOIP and are integrated with our Email systems for VMail access. All of the secured services inside our firewalls including network storage and other services can be access through our VPN firewall authenticated specifically to Emory faculty, staff, and students.

Currently, the computers for data access and analysis are at least an I7 CPU configuration with 8GB of RAM memory, and have CD/DVD-RW and 20" or higher flat panel monitors standard with 500 gigs local diskspace and 64 bit Windows. Our Apple Mac environments are generally iMac configurations or Powerbook laptops with at least 4 gigs of memory. All of our desktop and laptop systems are connected to our network storage array that provides both highly secure and open storage areas. The network storage array is around 255TB of useable space and subdivided between a secure areas and an open access area. Our student computing environments are provided through our RSPHDesktop environment. This is a Citrix based virtual desktop environment that provides over 40 plus applications to our students and is accessible from any place they can get on a network and open a browser. We also supply through our Citrix frame 10 open use high end computing desktops. These are available for out for researchers to access an equivalent of the analytical desktop computer through a browser

interface. This provides access to our full series of applications to a field deployed faculty member or one in temporary need of a high performance desktop environment in house. All desktop computers are configured based on best practices in industry as well as those outlined in NIST SP 800-69 Guidance for Securing Microsoft Windows XP Systems for IT Professionals: A NIST Security Configuration Checklist. All Windows machines have anti-virus software installed with updated virus signatures as well as the latest Microsoft XP Professional updates. The desktops required authentication from the Emory Active Directory to gain access to network services. In addition to the LINUX server used for reporting and analysis, there will be various applications that are PC-based. These include various MS files for word processing and graphics etc that are useful to share among the staff. The RSPH maintains a Windows NT based PC network. All shared PPG applications will be stored on a network drive.

Information security: The Rollins School of Public Health (RSPH) information technology environment is a HIPAA covered entity and complies with HIPAA and Emory information security and privacy policies and practices. In compliance with these policies and practices, RSPH aligns with the National Institute of Standards and Technology (NIST) special publications (800 series) for identifying, assessing, and managing information security risk within a technology environment. Drawing on federal and industry best practices, RSPH has implemented a series of multi-layered security controls to protect the integrity, reliability, and confidentiality of data. A sample of the key security controls includes:

- An annual risk assessment of all RSPH information technology assets with their level of risk, potential impact, probability, and controls evaluated based on NIST SP 800-30 Risk Management Guide for Information Technology Systems.
- RSPH and Emory network are protected by firewalls and intrusion detection devices. Rules on these devices are set to deny all traffic by default and "allows" are written as exceptions. These devices are updated as appropriate through Emory University's change management process and evaluated to ensure they provide the appropriate level of protection based on the sensitivity level of the data.
- Servers are housed within a secured network operating center (NOC). The NOC has environmental controls (fire, water, temperature), is accessible only through a two-factor authorization system (key card and passcode), and is accessible only by authorized information technology personnel. In the event of a power outage, the NOC devices will draw UPS power then from a backup generator.
- All servers are configured based on RSPH and Emory University best practices. Only authorized, trained system administrators have administrative privileges on the servers. System administrators monitor security mailing lists and sites and patch/update systems based on priority of the patch. All servers are periodically scanned for vulnerabilities and any identified vulnerabilities are assessed and managed.
- All information technology personnel go through background checks before gaining access to administrative privileges. At point of termination with Emory, all information technology personnel's administrative privileges are removed.
- Protected health information (PHI) data and the services that manage them are stored on separate network and server infrastructure with limited access and additional security controls.

  Data are backed up daily. Backups are stored in a tiered structure for disaster recovery purposes and include local, off-site, and out-of-state storage. Data stored off-site are encrypted to prevent compromise and can only be retrieved by authorized personnel.
- Data written to any RSPH file servers are checked with server-based anti-virus software. Access to data is verified with a local single point of contact within each department before any access control is granted. Principle Investigators are required to review access control lists each year to ensure continued accuracy.
- All RSPH desktops are configured based on best practices in industry as well as those outlined in NIST SP 800-69 Guidance for Securing Microsoft Windows XP Systems for

IT Professionals: A NIST Security Configuration Checklist. All Windows machines have anti-virus software installed with updated virus signatures as well as the latest Microsoft XP Professional updates. The desktops required authentication from the Emory Active Directory to gain access to network services.

- Security policies are created and reviewed through the Woodruff Health Sciences Center HIPAA committee, the Emory University Technology Infrastructure and Policy committee, and, local policies, through the RSPH Information Technology Advisory committee.

A full back up is performed every week with incremental backups nightly. Thus there is a 24 hour window of redundancy of our files. Also we use Raid 5 to protect the primary data drives that are backed up to a secondary set of drives and then moved to tape as the final backup. The tapes are encrypted and moved out of state for disaster recovery protection.

Statistical Methods:

Statistical methods: Circulating RBC volume and and RBC survival will be determined by flow cytometry as previously described [26-28] with the following modifications. The binding of fluorescent-tagged streptavidin to the biotinylated RBCs is terminated at 10 min by addition of 1 mL of 10 nM biotin in washing buffer to prevent cross-linkage of the heavily biotinylated RBCs with biotin binding sites on tetrameric streptavidin. Each population of biotinylated RBCs will be discretely enumerated using a histogram relating number of RBCs to the log of fluorescent intensity. For this analysis, up to six windows will be set separately by flowing separate samples of unlabeled blood and each of the different biotin density populations. For each population of biotinylated RBCs, "% biotinylated" will be calculated by dividing the number in that population by the number of total cells (biotinylated plus unlabeled). A standard curve will be constructed by sequential *in vitro* dilutions of each population into autologous normal RBCs. Initially, a simple paired t-test will be used to identify significant differences in cirulating b-RBC concentrations between different time point. Additionally, we are discussing more detaield analyses with Mr. Easley.

Number of subjects: 8 adults

Sample size rationale: Sample size was determined based on conversations with Dr. Jack Widness' group at University of Iowa, who pioneered this methodology. Rather than using a power analysis, since this is just a single-site single-arm study directed at validating the biotinylation methodology in our laboratory, the proposed recruitment of 8 subjects is based on Dr. Widness' estimate of how many repetitions of the procedure we should perform prior to moving onto the second phase of the study (not included herein), which is turn was based on his experience in working with the FDA to receive IND approvals for clinical trials with b-RBCs.

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