Official Title: **Red Blood Cell Survival Following Transfusion in Infants**

NCT00731588

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Modifications of Prior Protocol Amendment: Change in Protocol *"RBC Survival in Adults with Prior Antibody Response to BioRBCs" (BB-IND 12990 Serial Number 0008, submitted August 27, 2012)*

Note: Yellow highlighting indicates #0017 changes, i.e., since #0016 submission)

Summary Addressing Specific FDA CRF 21 Sec 312.30 Protocol Amendments: (b) Changes in a protocol.

This is a second revised protocol with minor changes replacing our prior Amended Protocol, "RBC Survival in Adults with Prior Antibody Response to BioRBCs" (BB-IND 12990 Serial Number 0008, submitted August 27, 2012 and its revision submitted July 19, 2016 as Serial Number 0016). Our goal as indicated below for this Phase 1 Study is to develop new knowledge that will improve the safety of administering BioRBCs to subjects who have developed anti-BioRBC antibodies following a prior BioRBC transfusion. We anticipate this is possible by administering BioRBCs in progressively increasing doses beginning with the lowest detectable 2.0 μg/mL BioRBC density ("BioRBC-2")—that we have been validating this past year—followed with subsequent progressive increases in BioRBC doses administered every 10-28 d. Since the July 19, 2016 protocol modification, we have completed study of our first adult study subject who received a single BioRBC dose of 2 μg/mL (results included below). In the current #0017 submission we propose a single minor modification of Protocol #0016 by combining the initial two lowest BioRBC doses in a single co-administration of both together to previously BioRBC sensitized adults, i.e., rather than as two separate low BioRBC doses separated by 10 to 28 d. As indicated in the Rationale section below, we anticipate that this minor modification remains a potentially safer protocol than administering all five BioRBC doses (i.e., BioRBC-2, -6, -18, -54, and -162) at a single time because:

(i) A lower total BioRBC dose is likely possible because the design of the protocol has been changed such that instead of the previous plan in our 2012 protocol amendment to give all five autologous BioRBC density doses in a single BioRBC transfusion (i.e., 2, 6, 18, 54, and 128 μg/mL all at once), the amended protocol calls for giving each of the five BioRBC density doses one at a time in progressively increasing density doses—now with the exception of the two lowest BioRBC density doses (2.0 and 6.0 μg/mL) which will be co-administered at the same time.

(ii) Although no new antibody testing procedure will be added to our previous 2012 protocol, we will continue to apply the more sensitive IgG agglutination detection assay we have developed to identify if and when BioRBC transfusion results in induction of plasma anti-BioRBC antibodies. Compared to even a year ago, we are significantly more experienced with and have developed improvement in this assay (1).

This modification is being concurrently submitted to our IRB. Our initial FDA Amended Protocol was approved by our local IRB in 2012.

A more complex Hypothesis (revised for #0017) that incorporates our latest observations. Upon re-exposure to autologous BioRBCs, adults who initially experienced an induced anti-BioRBC antibody response will demonstrate an anamnestic immune response; the threshold for this response may be at densities as low as BioRBC-6, or even -2. However, the resultant anti-BioRBC antibodies will not accelerate removal of the lowest BioRBC densities, i.e., BioRBC-2 and -6. Thus, this hypothesis tacitly invokes two immunological thresholds: 1) one threshold for the initiation of an anamnestic BioRBC antibody response; and 2) a second distinct and likely

higher threshold required for the macrophage immune system to recognize the antibody on the BioRBCs and thus accelerate their premature removal from the circulation.

Rationale. Pre-clinical and clinical studies of different antigen dose exposure suggest the presence of a threshold effect in the ability to elicit a specific antibody effect. Pre-clinical alloimmunization mouse models of humanized Kell antigen have demonstrated a threshold effect in pregnant dams (2). In addition to these mouse studies there are human data of partial RhD phenotypes suggesting different immune responsivity compared to RhD itself (3, 4). Consistent with these studies by others, adult subject 521 demonstrated a threshold-like dose effect *only* with the lowest of the four BioRBC densities that were concurrently administered in her subsequent BioRBC exposure (Study B) (**Figure 1**) (5). Specifically, Subject 521's pattern of BioRBC survival of the lowest BioRBC density (6 µg/mL) in Study B demonstrated markedly prolonged survival of ~100 d relative to that of the three much more rapidly removed higher densities (~7, 14 & 21 d for BioRBC-18 µg/mL, -54 µg/mL, and -162 µg/mL respectively)—despite administering the same number of BioRBC for each of the four BioRBC densities.

In September 2016, Subject 521 underwent a third Study C with the administration of only a single 2 µg/mL density dose of BioRBC (**Figure 1**). As anticipated: rather than being shortened, the survival of 2 µg/mL BioRBC was identical—indeed superimposable with—the first BioRBC study prior to the development of anti-BioRBC antibodies. What was also observed, but had not anticipated, was an early anamnestic anti-BioRBC antibody response beginning 5 d post-BioRBC administration (**Figure 2**). This finding led to modification of our hypothesis and to protocol revision #0017.

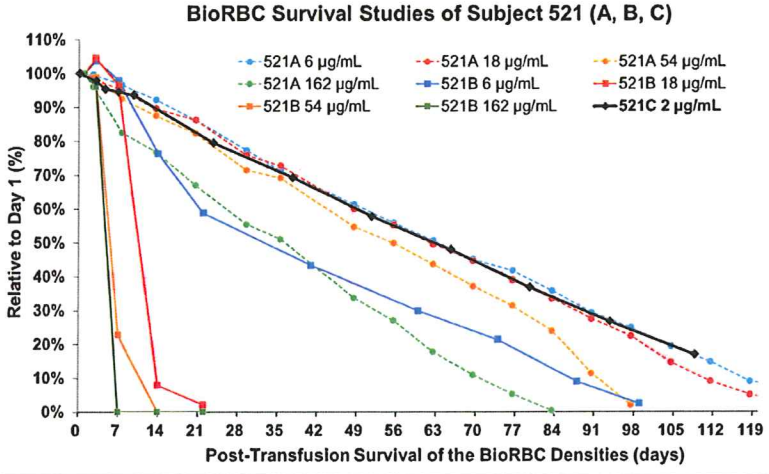


Figure 1. BioRBC survival in subject 521 who tested positive for anti-BioRBC antibodies (5). Dotted lines indicate the initial 2008 **Study A** survival of 4 BioRBC densities transfused: 6 µg/mL blue, 18 µg/mL red, 54 µg/mL orange, and 162 µg/mL green. Enrichment of each of 4 BioRBC densities was 0.6% of total RBC. Solid lines indicate the results of the 2nd 2013 **Study B**, showing accelerated removal of all but the lowest 6 µg/mL BioRBC density. The solid black line indicates the 2 µg/mL BioRBC survival of **Study C.**

Additional data suggestive of a BioRBC density threshold effect for anti-BioRBC antibody induction in humans are inferred from the fact that two of our collaborating groups who dosed 18 adult subjects with significantly lower doses of BioRBC using densities BioRBC-6 (6) and BioRBC-18 (7)—but failing to observe induction of anti-BioRBC antibodies in. All 18 adult subjects received much "lower dose" BioRBC transfusions, i.e., 10 mL packed RBCs/kg labeled at BioRBC density ~18 µg/mL, compared to the >1 log higher BioRBC dose our Iowa group administered: 6+18+54+162 = 240 µg/mL.

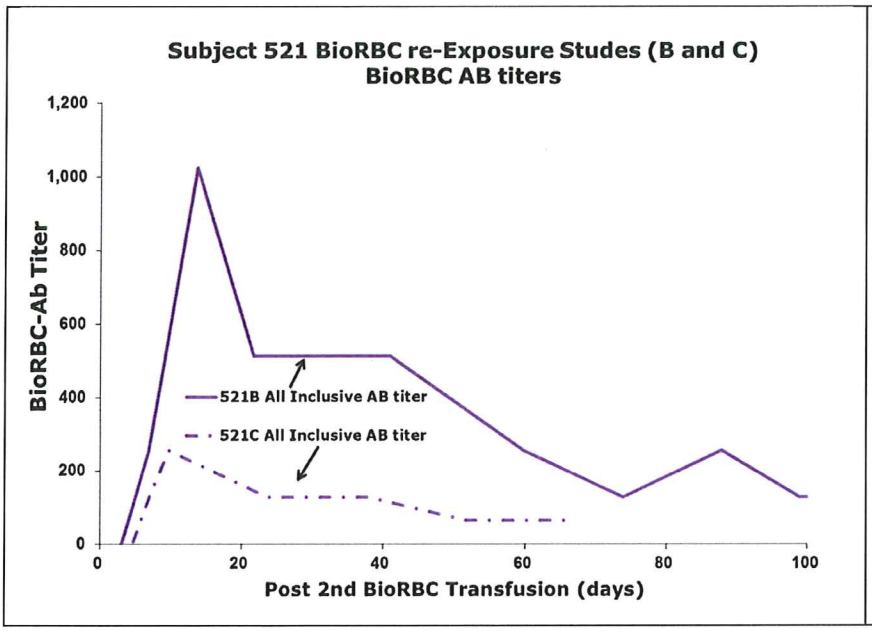


Figure 2. Comparison of anti-BioRBC antibody titers subject 521 following the second (Study B) and third (Study C) autologous BioRBC dosing. To provide greater uniformity, the antibody titers for each of these studies were measured in a single assay (but not all at the same time). In **Study B**, all four BioRBC densities (6, 18, 54, and 162 µg/mL) were administered at one time. In **Study C** a single very low dose BioRBC density of 2 µg/mL was administered. The much larger BioRBC antigen dose in Study B was associated with a greater anamnestic anti-BioRBC antibody response relative to Study C.

Background: Additional Details of Prior Studies by Investigators (unpublished)

In initial Study A following the administration of four autologous BioRBC densities (6, 18, 54, and 162 µg/mL), Subject 521 developed detectable anti-BioRBC antibodies 12 wk post-BioRBC dosing (5). In Study B Subject 521 was re-dosed with the same four BioRBC densities. Details regarding both Studies A and B were provided to the in our Annual FDA Report two years ago (Annual Report: BB-IND 12990, Serial Number 0014, submitted June 19, 2014, in a subsection entitled, "Results of adult subject re-exposed to BioRBCs following initially developing Abs"). Safety data reported in that report of Subject 521 documented no clinical change in the subject's prior good health included: BioRBC kinetics, BioRBC antibody titers, hematology laboratory results, biotin nutritional status, and general health. The only abnormality was the above noted rapid removal of the four densities of BioRBC transfused coinciding with an anamnestic anti-BioRBC antibody response (**Figure 1**). In the past 3 years, Subject 521 has remained in good health with detectable anti-BioRBC antibodies as measured in our IgG agglutination detection assay including an increase in anti-BioRBC antibody titer 5d after receiving her third BioRBC dose in Study C (**Figures 1 & 2**).

Subject 521's anti-BioRBC antibody titers did not appreciably change from one year after Study B BioRBC transfusion until three years later, i.e., antibody titers remained positive for plasma dilutions between 1:4 to 1:8 at BioRBC-54 target density). Following Subject 521's third 2 µg/mL autologous BioRBC administration in Study C an anamnestic antibody response was observed.

In addition to Subject 521, our University of Iowa research team has encountered other adult subjects previously exposed to autologous BioRBC who developed anti-BioRBC antibodies including:

- Three adults with definite, but weakly positive responses. These adults were tested using a tube agglutination antibody detection assay using a single BioRBC target density of 32 µg/mL (8). Unfortunately, there is no available plasma from these subjects reported in the 1990's (9) available to test in our current more sensitive (i.e., by >1 order of magnitude) IgG gel card anti-BioRBC antibody detection assay (1). These three adult subjects received ~50 mL of BioRBC density ~36 µg/mL. Although

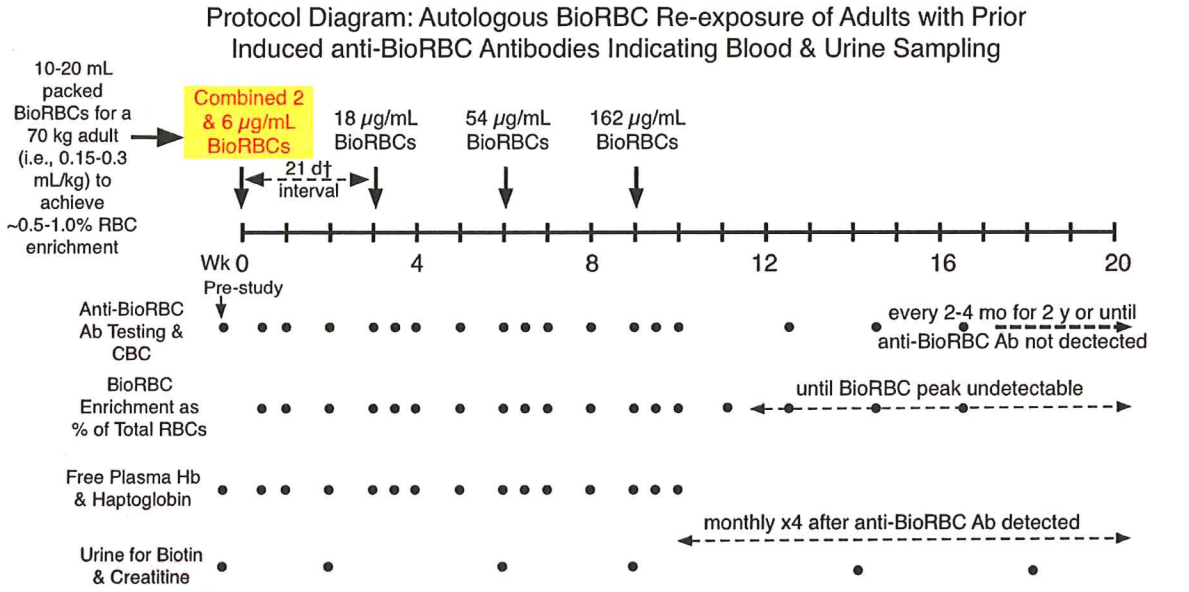
we have IRB permission to re-contact these subjects, the whereabouts of only one is currently known.

- Two adults that we more recently reported (10)—along with Subject 521—who demonstrated anti-BioRBC antibodies when tested in our more sensitive IgG gel card assay. In these 3 subjects anti-BioRBC antibodies were first detected at 12-16 wk post-BioRBC transfusion (11). (*Note*: Only the plasma of Subject 521 tested positive in both the tube and gel card assays; the other 2 subjects tested *negative* in the tube agglutination assay but positive in the gel card assay at BioRBC target densities ≤256 µg/mL.) In their initial BioRBC study ~3-4 y ago, subjects received ~50 mL of 4 biotin densities: 6, 18, 54, & 162 µg/mL. Although we have IRB permission to re-contact these subjects, we have not done so.

Thus, there are four potential adult subjects available at our Iowa site with induced anti-BioRBC antibodies from prior BioRBC doses, including Subject 521.

Study Protocol

In contrast to our previous protocol submitted in our Prior Protocol Amendment, "RBC Survival in Adults with Prior Antibody Response to BioRBCs" (BB-IND 12990 Serial Number 0008, submitted August 27, 2012), the current study protocol will be carried out in adult subjects in which increasingly higher BioRBC doses will be administered at 10-28 d (or longer as needed based on the availability of individual subjects) intervals as illustrated in this Protocol Diagram that includes the current #0017 modification of co-administering 2 and 6 µg/mL BioRBCs as the first dose:



† Although 21d study intervals are shown, the range of days between studies will be from 10 to 28 d (or longer), depending on individual subject availabity. ***NOTE***: Subsequent BioRBC studies will only be performed if anti-BioRBC antibodies are not observed.

This revised #0017 protocol modification was developed based on data from Subject 521 Study C presented above in the Rationale section. Although in all prior publications we have used the four BioRBC densities indicated in Figure 1, improvements in flow cytometry instrumentation and methods permit inclusion of a fifth density that is 3-fold lower than our previously lowest BioRBC-6 density, i.e., BioRBC-2. Consistent with our revised hypothesis, we predict that the co-administration of the two lowest densities (BioRBC-2 and -6) will elicit an

anamnestic anti-BioRBC antibody response but will not shorten BioRBC survival of either of these densities. We have elected to co-administer BioRBC-2 and -6, but not include BioRBC-18 in the first infusion mixture because: 1) in 521 Study B, the BioRBC-18 density demonstrated very rapid removal within 3-4 wk of dosing (**Figure 1**); and 2) if successful, 2-density dosing with BioRBC-2 and -6 will pave the way for concurrent determination of RCS in two populations of BioRBC (e.g., paired head-to-head comparisons, two RBC storage medias at the same time) even in sensitized subjects. Our rationale for continuing to include multiple sequential BioRBC dosing studies separated by 1-28 d is to more comprehensively address our mechanistic hypothesis because the hypothesis is primarily based on the results of a single subject (Subject 521). Other subjects may require greater BioRBC density doses to manifest an anamnestic anti-BioRBC antibody response.

Provided we are able to locate and enroll the four former study subjects who have developed anti-BioRBC antibodies, we anticipate that all four will experience anamnestic BioRBC antibody responses following re-exposure to autologous BioRBC. As indicated in the study protocol, we will administer progressively increasing "doses" of the same five BioRBC densities we have used previously in kinetic studies, beginning with the co-administration of BioRBC-2 and -6, followed by single BioRBC densities of BioRBC -18, -54, and -162. This will allow determination of when and to what degree an anti-BioRBC antibody response occurs, and what the resultant survival of the autologous BioRBC densities are. Particular attention will be paid to whether both BioRBC-2 and BioRBC-6 have similar long-term survival identical to those in their initial Study A. If BioRBC-2 and BioRBC-6 demonstrate the same, normal long-term survival, this indicates that *in vivo* long-term BioRBC survival kinetic studies using these two densities are reliable, even in the presence of anti-BioRBC antibodies. Although not proven by such findings, we further speculate that future RBC kinetic studies using BioRBC-2 and BioRBC-6 may be less likely to induce anti-BioRBC antibodies. The "dose" of BioRBCs administered is calculated based on the volume of autologous RBCs transfused and the biotin density on the RBCs. For example, if a 70 kg adult receives 15 mL of packed BioRBCs (i.e., ~0.6% of their circulating RBC volume assuming a blood volume of 80 mL/kg and a Hct of 45%) biotinylated at 6 μg/mL, then the biotin dose would be 6 μg of biotinylating reagent/mL x 15 mL = 90 μg/70 kg, or 1.29 μg of biotinylating reagent/kg. Based on this estimates, the co-administration of BioRBC densities of 2 and 6 μg/mL will be 56% less than the next higher dose of BioRBC-18. Testing for anti-BioRBC antibodies will be performed using IgG gel cards using the following three target-reagent densities: BioRBC-54, -162, and -256 μg/mL. As indicated in the Protocol Diagram, once anti-BioRBC antibodies are detected in the gel card assay, no further BioRBC doses will be administered. Based on Subject 521 Study C, we tentatively anticipate it is likely that the other three subjects with induced BioRBC antibodies will experience an anamnestic BioRBC antibody response following the initial co-administration of BioRBC-2 and -6.

As in our previous BioRBC studies, we will test each study subject for evidence of those laboratory parameters indicated in the Protocol Diagram above:

1. Anti-BioRBC antibodies & CBC: by IgG gel card detection assay on anticoagulated, whole blood samples beginning pre-BioRBC transfusion and post-transfusion at 3±1 d, 7±2 d, 14±2 d, and at 2 wk (±3 d) at subsequent intervals of every 2-4 wk up to 5 months post-transfusion. Complete blood counts (CBC) will be measured by the Sysmex Hematology Analyzer. Although based on our experience with Subject 521 we anticipate all subjects will have undetectable plasma anti-BioRBC antibody titers, subjects will still be studied if anti-body titers are ≤1:32 at target density BioRBC-

54. If as anticipated subjects demonstrate develop anti-BioRBC antibodies, they will be followed in the same manner as all subjects but thereafter be sampled 2-3 months for up to one to two years post-BioRBC transfusion. Based on experience with Subject 521, subsequent antibody titers, if present, do not change.

2. Flow cytometric analysis of BioRBC enrichment of autologous RBCs as determined by flow cytometry. This will be done on anticoagulated, whole blood samples drawn at the same intervals post-BioRBC transfusion as for anti-BioRBC antibody detection. Once BioRBC are no longer detectable, however, no further enrichment testing will be done. (The lower limit of detection is 0.06% of total RBC.)
3. Acute hemolysis similar to what we have previously reported in adult subjects: by determining free hemoglobin and haptoglobin levels in plasma samples drawn immediately prior to and at the following intervals following each progressively increasing BioRBC dose: 3±1 d, 7±2 d, 14±2 d, and at 2 wk (±3 d) intervals until the next subsequent progressively increasing BioRBC is needed, i.e., only if anti-BioRBC antibodies are not detected. In situations where anti-BioRBC antibodies are detected, blood sampling will be done for one month following the same protocol as for anti-BioRBC testing.
4. Biotin nutritional status prior to and following BioRBC transfusion. As done with Subject 51, spot urine samples will be obtained prior to study and immediately prior to each subsequent progressively increasing BioRBC transfusion. Once an antibody response is observed, spot urine sampling will be done monthly for 4 months, i.e., during the period of the anticipated highest anti-BioRBC antibody titer (**Figure 1**).

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