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Original Research

Development and Characterization of a Model for Inducing Fetal Hemoglobin Production in Cynomolgus Macaques (*Macaca fasicularis*)

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Hydroxyurea induces production of fetal hemoglobin (HbF), a tetramer of α and γ globin proteins and corresponding heme molecules, normally found in less than 1% of adult RBC. Increases in circulating HbF are correlated with clinical improvement of patients with hemoglobinopathies, and hydroxyurea, as a daily medication, is the standard treatment for sickle cell anemia. Although olive baboons (*Papio anubis*) are considered a key model species for HbF induction, cynomolgus macaques (*Macaca fasicularis*) are another species that conserves the ability to produce HbF into maturity. In this study, moderate anemia was experimentally induced in cynomolgus macaques by phlebotomy, to stimulate accelerated erythropoiesis and HbF production. In contrast to previous studies, vascular access ports were implanted for phlebotomy of conscious monkeys, followed by fluid replacement. As total Hgb levels dropped, reticulocyte counts and the percentage of HbF-expressing cells increased. Once total Hgb levels declined to less than 8 g/dL, 2 courses of oral hydroxyurea (once daily for 5 d) were completed, with a 9-d interval between courses. After hydroxyurea dosing, the percentage of HbF-expressing cells and total HbF were increased significantly. In addition, a significant but transient decrease in reticulocyte count and a transient increase in MCV occurred, replicating the characteristic response of patients receiving hydroxyurea. Daily clinical observations revealed no serious health issues or decreases in food consumption or activity levels. Methods were established for assessing the patency of vascular access ports. This study details a new protocol for the safe and routine induction of moderate anemia in cynomolgus macaques and validates its use in the investigation of novel pharmacologic entities to induce the production of HbF.

Abbreviations: SCD, sickle cell disease; HbF, fetal hemoglobin; VAP, vascular access port

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Fetal hemoglobin (HbF) is a tetramer of α and γ globin proteins that are expressed in human and NHP during fetal development through the first few months after birth. Infants then undergo a 'hemoglobin switch' to adult hemoglobin, which is composed of α and β globin proteins. HbF normally is only found in less than 1% of normal RBC and binds oxygen with greater affinity than adult hemoglobin.[5,6,14] Sickle cell disease (SCD) is a common heritable disease in humans. Mutations in the gene responsible for β globin result in abnormal hemoglobin that damages RBC. Resulting RBC have decreased lifespan in circulation and reduced cellular plasticity, which results in vasooclusion, causing tissue hypoxia and ischemia.[14] SCD can be painful, may involve acute chest syndrome episodes and multiple blood transfusions, and can cause mortality.[12,18] The only approved medicine for the management of SCD is hydroxyurea, which works by inducing the production of HbF through its cytostatic mechanism that selects for early progenitor cells after the death of late progenitor cells.[14] RBC that contain HbF are larger and more flexible than normal adult RBC, both of which characteristics increase their lifespan.[14] People with SCD typically have higher-than-normal HbF levels, likely due both to the need for increased erythropoiesis to compensate for sustained hemolysis and to their longer lifespan in circulation. Moreover hydroxyurea-induced increases of circulating HbF-positive RBC are correlated with clinical improvement.[2] To compare novel treatments with hydroxyurea, the current standard of care, a preclinical model of HbF induction is needed. Because the induction of HbF production by using hydroxyurea is reported to require a state of stress erythropoiesis due to chronic anemia typical in SCD patients,[11] we used repeated phlebotomy to induce moderate anemia in our model.

Myelosuppression and inconsistent responses in both adult and young patients limit the utility of hydroxyurea in humans.[12,18] Therefore, safer and more widely efficacious treatments are needed urgently. Although a transgenic mouse model of SCD is available,[17,19] important in vivo models for HbF induction are NHP-based, due to the evolutionary conservation of the γ-globin genes that are unique to HbF and which are present in humans and some NHP species.[14,21] Both baboons and cynomolgus macaques retain hemoglobin switching, which is not present in other nontransgenic laboratory animals.[11,22] In addition, the time of differentiation from erythroid burst-forming units to mature erythrocytes is more similar between humans (14 d) and cynomolgus macaques (16 d) than between humans and mice (7 d); this similarity may result in similar times to treatment

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effects.[16,22] Finally, hydroxyurea is essentially ineffective at inducing HbF in various transgenic mouse models of SCD[9] but has been shown to reliably induce HbF in a baboon model.[8] The molecular target is conserved in cynomolgus macaques, which potentially are a more appropriate species than baboons because cynomolgus macaques are more widely used than baboons and are a better bridge to future safety assessment work.[4]

Materials and Methods

All procedures were conducted in accordance with the GlaxoSmithKline Policy on the Care, Welfare, and Treatment of Laboratory Animals and were reviewed by the IACUC at GlaxoSmithKline.

Animals. Healthy male cynomolgus macaques (*Macaca fasicularis*; *n* = 3; age, 4 y; weight, 4.2 to 5.1 kg; Covance, Alice, TX) were housed in an AAALAC-accredited facility at GlaxoSmithKline (Collegeville, PA) throughout the study. These animals were housed indoors, maintained on a 12:12-h light:dark cycle, fed a standard primate diet (Certified Primate Diet 5043, LabDiet, St Louis, MO) that was supplemented daily with a variety of fresh foods and forage items, and had unrestricted access to water. The macaques were provided with toys and auditory or visual enrichment daily. Prior to study enrollment, they underwent physical examination, baseline bloodwork (CBC and chemistry panels), and training, which included acclimation to pole, collar, and chair restraint. Behavior during these activities was assessed to select animals amenable to this type of study work.

Surgery. Ipsilateral arterial and venous vascular access ports (VAPs) were implanted aseptically in anesthetized animals by using 3.5-French silicone rounded tip-catheters in the femoral artery or vein and were connected to titanium domes placed subcutaneously in a pocket created on the animal's dorsum. The VAP system was checked for patency and locked by using sterile heparin–glucose solution (Cath-Loc HDS, SAI Infusion Technologies, Lake Villa, IL). This procedure has been described in detail elsewhere.[20] Appropriate analgesics (butorphanol and flunixin meglumine) were provided preoperatively and postoperatively. Macaques were assessed for pain and incisions were monitored until healing was complete. Study activities began no sooner than 4 wk after surgery.

Phlebotomy. VAP were accessed aseptically 3 times each week (Monday, Wednesday, and Friday) for phlebotomy followed by fluid replacement; during these procedures, macaques were conscious in chairs, and a mirror and toys were available. As previously described,[20] the area over both ports was clipped and prepared aseptically. A Huber needle was inserted through the skin into the subcutaneous port. Lock solution and a small amount of blood were withdrawn and discarded prior to the collection of any samples. Phlebotomy was performed by using either 3- or 5-mL syringes until the entire volume was collected, usually from the arterial VAP. This sample was the source for all blood tests described. Phlebotomy volumes ranged from 5 to 10 mL/kg, with the volume adjusted up or down according to the current Hgb level and a target range of 6.5 to 8.0 g/dL. An equal volume of sterile saline was infused into the venous VAP after phlebotomy to replace the volume of fluid lost.[1] Both catheters were flushed with saline and locked by using a heparin–glucose lock solution. Correct aseptic access of port domes for phlebotomy was maintained throughout the process to minimize the risk of infections, septicemia, and bacteremia. The percentage of HbF-positive RBC and total Hgb levels were measured 2 or 3 times each week.

Assessment of the effects of phlebotomy and hydroxyurea treatment. During and after phlebotomy procedures, macaques were closely monitored for any evidence of weakness. In addition, the veterinary staff performed health status checks twice daily on phlebotomy days and once daily on other days. Food intake and fecal and urine output levels were tracked daily. Body weights were recorded weekly.

CBC analysis (including total Hgb-positive cells) and reticulocyte counts were performed 2 or 3 times each week by using a flow-cytometry–based system (Advia 2120i hematology analyzer, Siemens Healthcare Diagnostics, Tarrytown, NY) using light scatter, myeloperoxidase and oxazine 750 staining. Blood chemistry values were monitored weekly by using an automated chemistry analyzer (model AU680, Beckman Coulter, Brea, CA). Iron levels (that is, serum iron, total iron-binding capacity, and percentage saturation) levels were assessed (Roche–Hitachi Modular D Analyzer, Roche Diagnostics, Indianapolis, IN; Animal Health Diagnostic Center, Cornell University, Ithaca, NY) weekly.

The percentage of HbF-positive cells was measured by flow cytometry (as a percentage of all blood cells) using Guava EasyCyte 8HT Cytometer (EMD Millipore, Darmstadt, Germany) after intracellular staining of whole blood with a FITC-labeled mouse antihuman HbF monoclonal antibody (Invitrogen, Camarillo, CA). In addition, the percentage of HbF as a proportion of total Hgb (that is, HbF / [HbF + HbA]) in whole-blood lysates was measured by using HPLC based on differences in retention times for HbF and HbA. Blood lysates were prepared by diluting the blood cell pellet in water, followed by high-speed centrifugation and frozen-storage of supernatants until analysis (D10 Analyzer, BioRad, Hercules, CA). Multiple time points (before hydroxyurea, after course 1, and after course 2) were analyzed.

Iron supplementation. To prevent concurrent iron-deficiency anemia,[13,21] the monkeys were given weekly intramuscular injections of iron dextran (Iron Dextran Injection USP, Watson Pharma, Corona, CA)]. The following equation from the package insert was used to calculate supplementation: iron replacement (mg) = weekly blood volume loss (mL) × Hct (from the previous phlebotomy). The first supplement given was a smaller volume (that is, based on 1 day's loss) and was monitored carefully, to prevent adverse reactions.[21] Supplementation was sometimes increased or decreased according to weekly iron levels in the context of baseline iron levels to ensure that over- or undersupplementation did not occur (data not shown).

Hydroxyurea dosing. Capsules containing 500 mg hydroxyurea (American Health Packaging, Columbus, OH) were opened and the contents dissolved in sterile water for oral dosing. When total Hgb levels were in the target range, oral dosing of 100 mg/kg hydroxyurea once daily for 5 d (2 courses total, with a 9-d interval between courses) was initiated. The phlebotomy schedule (at 7.5 mL/kg) was maintained during dosing.

VAP assessments. Every time VAP were accessed for phlebotomy, the degree of function was noted. When a VAP was nonfunctional, a thorough assessment was completed. Assessments included survey and contrast radiographs of catheters and VAP domes, followed by ultrasonography with and without the use of contrast agents. Macaques were sedated with ketamine (10 to 15 mg/kg IM; Ketaset, 100 mg/mL, Fort Dodge Animal Health, Fort Dodge, IA) followed by dexmedetomidine (0.01 to 0.03 mg/kg IM; Dexdomitor, 0.50 mg/mL, Pfizer Animal Health, New York, NY). Ports were prepped as previously described, and contrast agent (MD76R, diatrizoate meglumine and diatrizoate sodium injection USP; Mallinckrodt, Hazelwood, MO)

was injected directly into the ports to evaluate for function by using radiographs. Lateral and ventrodorsal positioning was used for radiography and ultrasonography. Microbubbles (Optison, GE Healthcare, Princeton, NJ) were used as the contrast agent for ultrasound assessment at the area of the catheter tip within the vessel. During these assessments, the first 3 mL of lock solution and blood that was removed aseptically from each VAP was immediately placed into a sterile blood culture vial (VersaTREK REDOX 1, Thermo Scientific, Lenexa, KS) for culture, to rule out infection.

Statistics. Data analysis was performed by using Incyte (version 2.7; EMD Millipore) and FlowJo (version 10.1, TreeStar, Ashland, OR). Statistical analysis for percentage of HbF-positive cells was performed by using SAS for Windows (version 9.3, SAS Institute, Cary, NC). Statistical analysis for HPLC data was performed by using one-way ANOVA with Tukey posttest (version 5, Prism, GraphPad Software, San Diego, CA). For all statistical tests, significance was defined as a P value of less than 0.05.

Results

Effects of phlebotomy and treatment with hydroxyurea. Subjective assessments of the macaques during and after phlebotomy revealed no adverse effects except for one occasion when a macaque appeared to be weak (quiet demeanor in an otherwise active monkey) with pale-pink mucous membranes. The animal was observed closely, recovered quickly after being placed into his home cage, and required no additional treatments or interventions. All other observations and parameters, including food intake and fecal and urine output, were within normal limits. The animals maintained body weight throughout the study, and no clinically significant changes in serum chemistry values were obtained (data not shown).

After the initial phlebotomy regimen (2 wk of 5 mL/kg 3 times each week), total Hgb had not reached the 6.5 to 8.0 g/dL range (Figure 1). Increasing the collected volume to 10 mL/kg 3 times weekly for 2 wk resulted in consistent values of total Hgb in the target range for all 3 macaques. In the subsequent 2 wk, all animals were transitioned to a maintenance protocol, in which anemia was maintained by phlebotomy at 7.5 mL/kg 3 times weekly. The maintenance phlebotomy protocol was continued throughout the remainder of the study. During courses of hydroxyurea treatment (course 1, days 49 through 53; course 2, days 63 through 67), blood was drawn prior to hydroxyurea dosing on days when both procedures occurred. Total Hgb decreased over time from a baseline mean of 13.9 g/dL to an anemic baseline mean of 7.3 g/dL during the week preceding hydroxyurea treatment. Corresponding with the decrease in total Hgb due to phlebotomy was a marked increase in the percentage of reticulocytes (Figure 2). No consistent change in MCV was noted during the pretreatment phase (Figure 3). The percentage of HbF-positive cells increased for all animals from original baseline (nonanemic) to a higher baseline (anemic) (Figure 4 and Table 1). As in human patients with SCD, the percentage of HbF-positive cells showed both daily variability and considerable intraindividual variability.

Total Hgb in all animals declined from day 1 to day 48 (the time period for characterization of anemia prior to dosing with hydroxyurea) during the Monday–Wednesday–Friday phlebotomy schedule. Decreases were consistently seen after every second day (Monday to Wednesday, Wednesday to Friday) during both phlebotomy-only and phlebotomy-plus-hydroxyurea treatments, with frequent increases during the 3 d (Friday to Monday) between phlebotomies, likely because of the longer

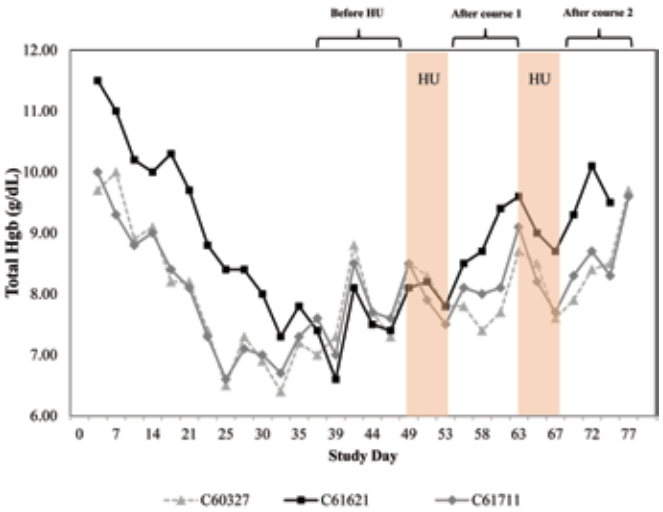

Figure 1. Total Hgb levels throughout the study for each macaque, starting on day 4 (day 1 is the first phlebotomy day). Bars indicate the 2 courses of treatment with hydroxyurea (HU).

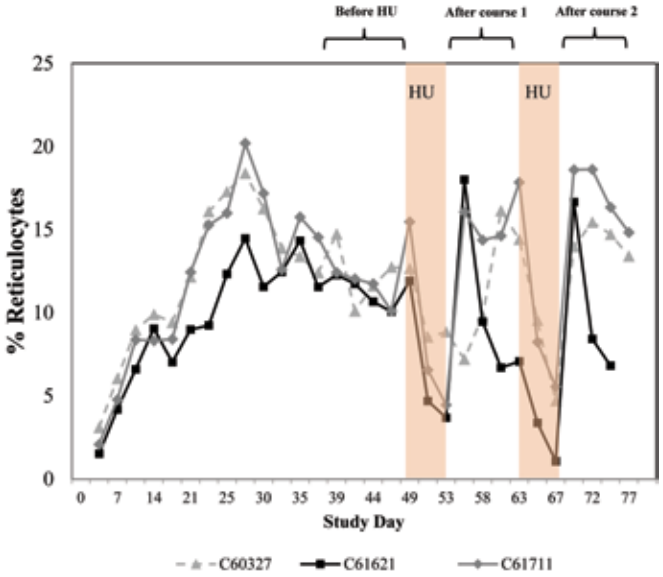

Figure 2. Percentage of reticulocytes for each animal throughout the study. Bars indicate the 2 courses of treatment with hydroxyurea (HU).

time period for RBC production between the Friday to Monday time points as compared with Monday to Wednesday and Wednesday to Friday (Figure 1). Reticulocyte percentage increased from day 1 to day 48 and decreased during both courses of hydroxyurea treatments. Both total Hgb and reticulocyte percentage then increased during the subsequent no-treatment weeks (Figure 2). Levels of neutrophils fluctuated within normal limits throughout the entire study (data not shown), and hydroxyurea treatment did not cause significant neutropenia (fewer than 1.5×10^9/L). Consistent with a common clinical effect of hydroxyurea,[14] MCV increased during the weeks after both treatment rounds (Figure 3). The percentage of HbF-positive cells was significantly ($P < 0.01$) increased after both courses of hydroxyurea as compared with pretreatment values (Figure 4 and Table 1).

To further confirm the effect of hydroxyurea on HbF levels, we used a validated HPLC method to measure alternative forms of Hb.[8] We observed a cycle-dependent increase in HbF after

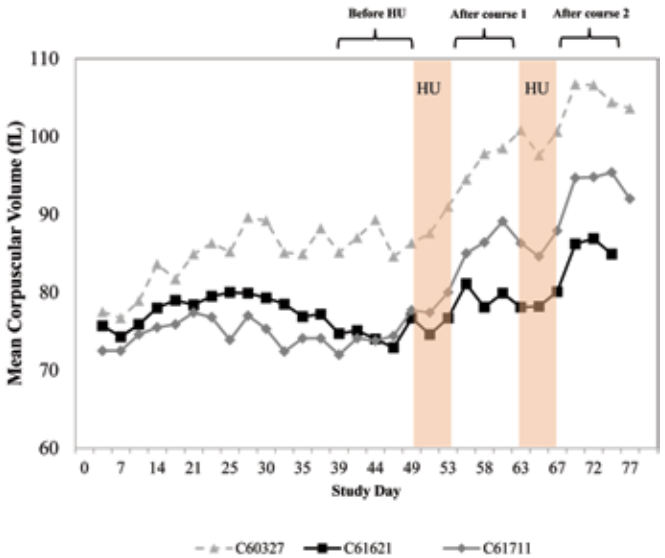

Figure 3. MCV for each animal throughout the study. Bars indicate the 2 courses of treatment with hydroxyurea (HU).

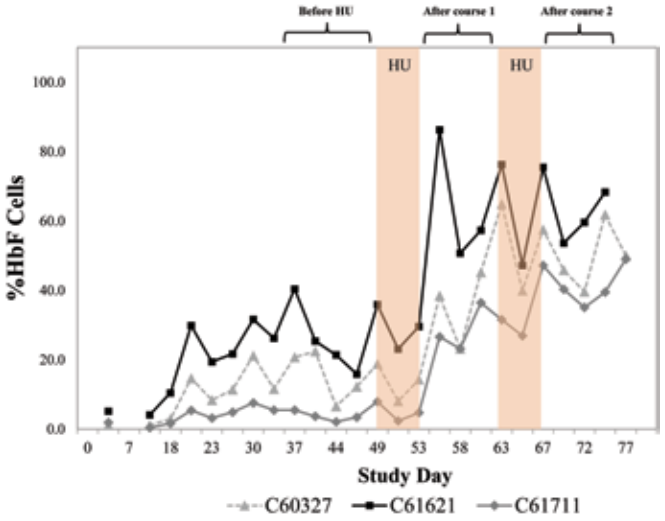

Figure 4. Percentage of HbF-positive cells for each animal throughout the study. Hydroxyurea (HU) induced significant increases in the percentage of HbF-positive cells. Brackets indicate time points used in statistical comparisons.

hydroxyurea treatment, and the increases were significantly ($P < 0.001$) greater after the second cycle of dosing (Figure 5).

VAP assessment. Findings from imaging procedures included faulty positioning of the catheter (Figure 6), leakage between the catheter and the port, and intermittent occlusion due to intravascular tissue around the catheter tip, likely fibrinous in origin (Figure 7). The decreased ability to collect blood from the VAP catheters likely was due at least in part to these problems. Culture results were negative.

Discussion

In this study, we developed a model of experimentally induced anemia by using phlebotomy to stimulate 'stress erythropoiesis' in cynomolgus macaques. This procedure establishes a state of accelerated erythropoiesis that models some aspects of SCD and that is critical to pharmacologic increases in HbF. Previous studies testing hydroxyurea and cytotoxic agents including vinblastine and 5-azacytidine have established the importance of accelerated erythropoiesis on the extent of pharmacologically induced HbF production in NHP.[11] For male cynomolgus macaques, Hgb levels of 6.1 to 8.6 g/dL (approximately 30% to 45% reduction from baseline Hgb) and Hct levels of 20.3% to 28.9% are consistent with moderate anemia.[1] Published literature for cynomolgus macaques suggests that 3 or 4 phlebotomies of 17 mL/kg each week will reduce Hgb levels to approximately 6.5 g/dL, and removal of 100 mL weekly will maintain these reduced Hgb levels.[10,11] For a 4-kg cynomolgus macaque this volume would represent approximately 26% of total blood volume. We, therefore, attempted to reach and maintain a target Hgb of 6.5 to 8.0 g/dL through regular withdrawals of smaller volumes. This approach draws on similar methods described to achieve moderate anemia in baboons.[8] Another study in a baboon model using indwelling catheters and a jacket–tether system involved daily phlebotomy of 2.5 to 5 mL/kg in animals weighing approximately 5 kg and aimed for Hgb levels of 6.5 to 7.5 g/dL.[15]

In refining our model, we developed a procedure that defined the length of phlebotomy necessary to induce moderate anemia, the volume of blood to withdraw per kilogram of body weight and the required frequency of phlebotomy, fluid replacement volumes, and the utility of weekly iron supplementation dosages. We have given examples of immediate changes that can be expected in total Hgb levels during large-volume bleeds during a baseline state and during anemia. Neither clinical observations nor intense monitoring of bloodwork revealed any adverse effects of our procedures over several months, leading us to believe that these withdrawn blood volumes were relatively benign for the animals and their wellbeing. Those observations notwithstanding, we conclude that close monitoring through both clinical assessments, CBC analyses, and serum iron values are highly recommended.

The 10-mL/kg volume is approximately 16.7% of the total blood volume when estimated as 6% of body weight[1] or is 14.3% of the total blood volume when body condition is considered.[7] A recent study recommended the removal of no more than 15% of the total blood volume as a single collection per week in light of episodes of vomiting or anorexia at 17.5% blood withdrawal volumes;[1] however our macaques did not demonstrate any of those adverse effects at our limit of 14.3% or 16.7% at each of 3 phlebotomies per week.

Importantly, our macaque model recapitulates many of the hematologic features of hydroxyurea treatment in patients with SCD. Patients who are managed with hydroxyurea frequently experience decreases in disease-elevated reticulocyte and neutrophil counts (indicative of myelosuppression) and increases in Hgb and MCV that correspond with rises in the percentages of HbF and HbF-positive cells.[14] In our studies, we observed marked changes in all of these parameters. In humans, when neutrophil counts are below 1.5×10^9/L or platelets are fewer than 100×10^9/L, treatment with hydroxyurea is discontinued until values return to initial parameters.[12,18] In addition to hemoglobin alterations, changes to hematologic parameters (reticulocyte counts, neutrophil counts, MCV) were all consistent with observed clinical effects of hydroxyurea treatment.[14]

In the absence of hydroxyurea treatment, we detected very low levels of HbF by HPLC (1% to 2% of total Hgb in all animals; Figure 5), with low and variable numbers of HbF-positive cells detected by flow cytometry (Figure 4). With hydroxyurea treatment, the proportions of both HbF and HbF-positive cells increased. After the second cycle of hydroxyurea, we observed modest increases (for animals C60327 and C61711) or no change (C61621) in the percentage of HbF-positive cells, but HPLC

Table 1. Effect of hydroxyurea on the percentage (%) of HbF-positive cells in cynomolgus macaques

		Course 1		Course 2	
Animal	Before (%)	%	*P*	%	*P*
60327	15.0 ± 3.5	42.9 ± 8.6	<0.01	49.3 ± 4.7	<0.001
61621	24.6 ± 4.2	67.7 ± 8.3	<0.001	60.5 ± 4.3	<0.001
61711	4.3 ± 1.3	29.5 ± 2.9	<0.01	41.0 ± 2.9	<0.001

The percentage of HbF-positive cells (mean ± SEM) before hydroxyurea treatment (days 39–49) was compared with that after each course of treatment (course 1, days 56–63; course 2, days 70–77). Data were compared within each animal, with 4 values per macaque per cycle. Significance was defined as $P < 0.05$ (2-way ANOVA)

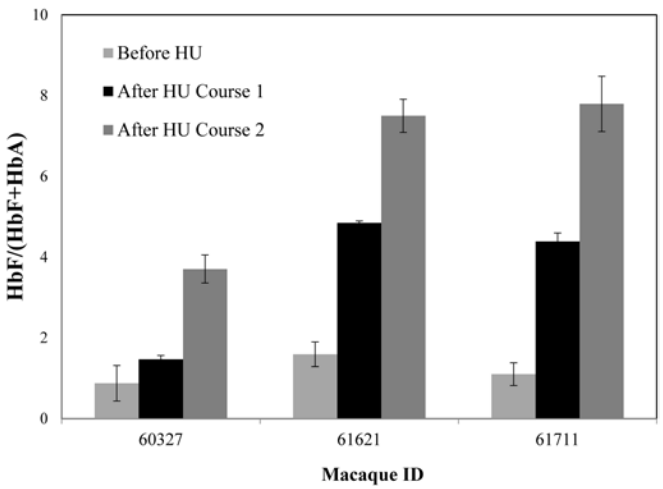

Figure 5. Comparison of proportional HbF levels (that is, HbF / [HbF + HbA]) before treatment with hydroxyurea, after treatment course 1, and after course 2. Data are given as the mean ± 1 SD of 3 time points. Hydroxyurea treatment resulted in highly significant ($P < 0.001$) increases in the rate of HbF levels after course 1 (2 of 3 macaques) and course 2 (all 3 animals). Noteworthy is the significant ($P < 0.001$) difference between courses 1 and 2.

revealed further increases in amount of HbF in all 3 macaques. These findings suggests that the amount of HbF produced per cell increased even when the number of HbF-positive cells did not.

Regarding long-term functionality of VAP, we continue to refine the surgical method, our procedures, and upkeep of the VAP system. Large-volume withdrawals of blood may cause excessive force on and increased resistance to the catheter tips which, over time, might explain the difficulty in obtaining the volumes required. This difficulty was unexpected, given that other VAP-implanted macaques in our colony did not demonstrate this level of decreased functionality. However, we believe the difficulty in our current model was due primarily to the frequency and volumes withdrawn. In this study, VAP failure occurred between 65 to 267 d (average, 138 d) after implantation. When one VAP failed, the remaining VAP was used for both phlebotomy and fluid replacement, allowing the study to continue with minimal delay.

Later surgeries were modified to improve the long-term success and patency of the VAP. These revisions included increasing the catheter size to 5 French and intraoperatively confirming correct placement of the catheters by using ultrasonography. Unfortunately, these modifications did not markedly improve the longevity of the VAP in our high-volume application (data not reported). Other adjustments to the phlebotomy procedure that we considered included using different-sized syringes, a luer access port attached to the Huber needle instead of a 3-way stopcock, and saline flushes when bloodflow slowed.

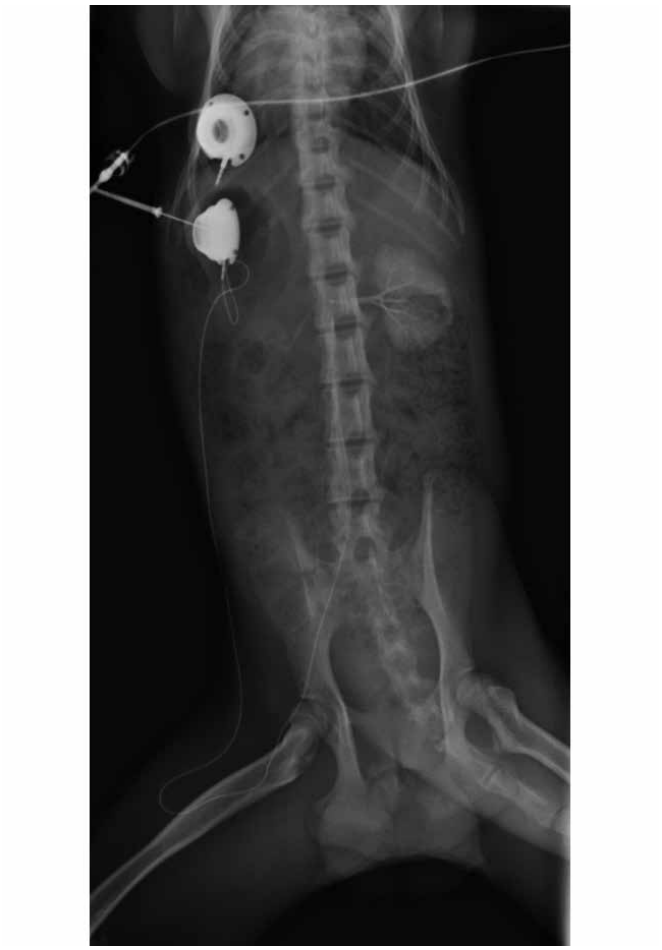

Figure 6. Arterial VAP entering the renal artery, a site cranial to the optimal position.

One limitation of this study is the low number of animals used to develop this model. In consultation with a statistician and in an effort to appropriately minimize the number of macaques used, 3 animals was determined to be sufficient to show a meaningful biologic response as proof-of-concept for this model and allowed for serial use of animals, with data collection before and after anemia and in response to hydroxyurea and—potentially—novel compounds so that all of those responses can be compared on an individual animal basis. Future work may expand these results by evaluating more animals, to fully validate this model.

This model may influence the identification of new potential therapeutic agents for the treatment of hemoglobinopathies. A similar model in anemic baboons has been used to test multiple pharmacologic inducers of HbF that subsequently showed different degrees of HbF induction in clinical studies.[8,15] The

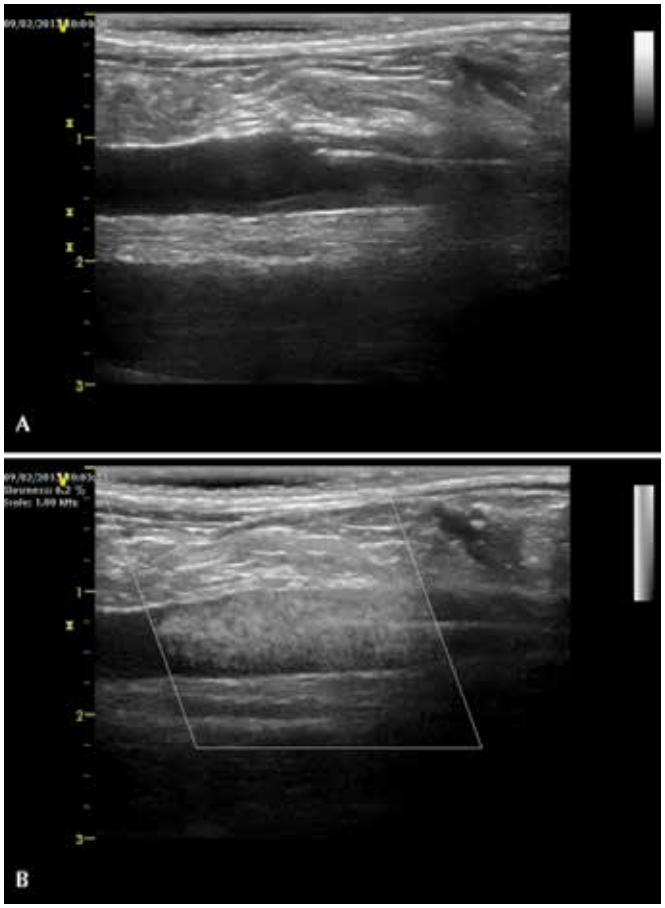

Figure 7. (A) View of catheter within the vena cava, before using microbubbles contrast agent. (B) Microbubbles entering vena cava from catheter, demonstrating intermittent tissue occlusion at the catheter tip.

current cynomolgus macaque model allows the determination of a pharmacokinetic–pharmacodynamic relationship for advanced test agents, enabling studies to better define dose selection for the safe and effective dose route and schedule in a more physiologically relevant system than the various transgenic mice available. Moreover, all procedures were well tolerated, and our macaques did not require sedation during phlebotomy. The unavailability of such a model risks inadequate consideration of benefit–risk scenarios and the inability to improve on existing treatments.

In summary, we have developed a reliable, easy-to-use in vivo anemia model in cynomolgus macaques, a species phylogenetically similar to humans, that can be used to develop and test compounds for the treatment of SCD. In addition to testing treatments for hemoglobinopathies, this model could be used to study other diseases that require an erythropoietic response, such as bone marrow disorders. As part of the model development, our refinements have used a new method of phlebotomy to create an anemic state. Our model is less invasive than others currently available and lacks potential variables such as ketamine sedation, which is known to affect many blood values.[3] The use of VAPs has a positive effect on animal welfare—a refinement consistent with our commitment to the 3Rs principle of animal research.

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