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Predictors of hemoglobin variability in a population of weaning age (three-to four-month old) rhesus monkeys

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Abstract

Sources of variability in hemoglobin concentration in blood were examined in over 600 rhesus infants at the California National Primate Research Center who had complete blood counts (CBCs) conducted at three-to-four months of age. These infants were born and raised in outdoor social housing. Hemoglobin values ranged from 8.5 to 15.3 µg/dL with a mean and standard deviation of 12.2 ± 0.8 µg/dL. As expected, hemoglobin was strongly associated with the number of red blood cells (RBCs). Plasma protein concentration, an indicator of blood volume, was not a predictor. Associations with infant age, weight and sex, infant serum cortisol, dam's reproductive history, and birth year, month and location were evaluated in regression analyses. Cage of origin, maternal age at delivery and infant weight were associated with hemoglobin concentrations. Unexpectedly, serum cortisol, determined at the same time as CBC samples were taken, was the strongest predictor of hemoglobin concentration. The basis, as well as the functional significance, of the variation in infant hemoglobin and its association with serum cortisol in this population of rhesus fed a nutritionally optimized diet and housed under standard conditions is relevant to the development of both nonhuman and human primate infants.

Keywords

rhesus infants; hemoglobin; cortisol

INTRODUCTION

Iron deficiency anemia (IDA) occurs at a high rate in human infants after weaning and is recognized as an important risk factor for impaired brain development in human populations [Georgieff, 2011]. Post-weaning IDA is also reported in macaque colonies maintained with commercial diets [Anderson et al., 1983; Bicknese et al., 1993; Kriete et al., 1995] and the relationship between developmental iron deficiency anemia and brain development is currently being investigated in controlled experiments in rhesus monkeys [Golub, 2010; Lozoff, 2011]. Some factors associated with weanling IDA have been identified in studies of rhesus infants [Coe et al., 2007; Lubach and Coe, 2006], but systematic population-based

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studies of anemia and iron status in macaques in managed colonies are limited to older populations [Bonfanti et al., 2009; Ganesh et al., 2009; Wolford et al., 1986]. Hemoglobin is a major index of iron status and IDA. While anemia is a common target for clinical intervention, variations in hemoglobin above the iron deficiency threshold are not commonly considered relevant to health and underlying origins of variation are not often studied.

The California National Primate Research Center (CNPRC) conducts a Biobehavioral Assessment (BBA) program for most three- to four-month-old rhesus in the colony to aid in colony management and basic research issues. This population represents the youngest age at which infants separate from their mothers and begin the weaning process [Lindburg, 1971]. As part of the BBA program, a blood sample for a complete blood count (CBC) is obtained. These data were available in the BBA database. The BBA database, along with electronic colony records at the CNPRC, allows hypothesis-based inquiries. In this study we hypothesized that maternal background, infant characteristics and environmental factors contribute to the variability of hemoglobin in this population.

METHODS

Assurance of compliance with animal codes

This research used data previously generated by the BBA program at CNPRC. The BBA protocol follows the Guidelines for the Care and Use of Laboratory Animals of the National Research Council [National Research Council, 2011] and the Principles for Ethical Treatment of Animals of the American Society of Primatologists. The BBA protocol was approved prior to implementation by the UC Davis Institutional Animal Care and Use Committee (IACUC).

Housing and husbandry

Infants in this study were born and raised outdoors in 18 half-acre field corrals. The corrals hold family groups of 65–180 animals per cage. They are outfitted with A-frame structures for perching and protection from the sun and rain and climbing structures for exploration and exercise. Radiant heaters provide supplemental heat when temperatures drop below 45°F. Water is available ad libitum from spigots in the cages. Commercial monkey diet and various enrichment food items are provided twice daily. During the years surveyed for the current report, the diet used was Lab Diet 5038 (Purina Mills Intl., St. Louis, Missouri, USA) containing 230 parts per million (ppm) iron. Trained technicians perform daily health checks. The corrals undergo regular preventive health checks including weights, tuberculin tests, vaccinations, physical exams, tattooing, and required blood samples. All animals are individually identified and a complete record of their health and reproductive activities is maintained in an electronic database.

Biobehavioral Assessment Program

The BBA Program was initiated at the CNPRC to identify characteristics of young infants that would impact the effectiveness of husbandry and management procedures. A blood

sample for CBC and serum cortisol was taken from all infants participating in the BBA program.

Briefly, for blood sampling in the BBA, infants and dams in the corral cages are captured together without sedation and the infant is separated for transport to indoor single caging where a series of behavioral evaluations will be conducted over a 25 hour period. A maximum of eight infants are captured and entered into the BBA evaluation at one time. About two hours after transfer, weighing, and an initial observation period, a blood sample is obtained without anesthesia for CBC and cortisol assay. The cortisol value is obtained as an index of the response to the stress of separation and relocation and would also reflect the stress of restraint and venipuncture. The results of the CBC and the cortisol assay, along with the behavioral data, are made available in spreadsheet format. In addition to the BBA database, electronic colony records provide information on the dam's reproductive history and the date and location of the birth.

BBA evaluations are conducted for infants from four rearing environments: outdoor half-acre corral cages, outdoor small group "corn crib" cages, indoor housing with dam and sometimes another dam and infant, and indoor housing in the primate nursery. Hematology values have been shown to vary with rearing environment [Capitanio, 2006]. For this study, only data from outdoor corral infants (the largest group) were included in the analysis. Infants from pregnancies born in 2002–2004 were used because there were no changes in the diet or the equipment used for CBC analyses during this three-year period. Hematology data from earlier years has been reported previously [Capitanio, 2006]

Blood sample assays

CBCs performed by the CNPRC clinical laboratory using ABX Pentra60™ (Horiba Medical, Irvine, California, USA) provided values for the following red cell parameters: red blood cells (RBCs), hemoglobin (Hgb), hematocrit (Hct), mean corpuscular volume (MCV), mean cell hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC). Plasma protein, an index of dehydration, and sample color, an index of hemolysis, were also provided. While blood cells (WBC) were determined as part of the CBC and CD4/CD8 lymphocyte ratios were obtained via flow cytometry (BD Bioscience FACSCalibur™, San Jose, California, USA). Serum cortisol was measured in plasma by radioimmunoassay (RIA) (Diagnostic Products Corp., Los Angeles, California, USA).

Data analysis

CBCs and colony record data were screened for biologically improbable outliers which were then evaluated for possible error and eliminated when appropriate. Three infants with hematocrits greater than or equal to 50% were considered dehydrated and were eliminated from the dataset. Any animal with missing data on any RBC parameter was eliminated from the sample. Potential hemoglobin covariates were evaluated with multiple regression using least squares or general linear modeling (JMP® Software, SAS Institute Inc., Cary, North Carolina, USA). An effect screening procedure provided by the software was used which queried the data for relevant hemoglobin covariates in separate analyses with infant, maternal, and environmental (year, month, cage) factors. The effect screening provided

graphical comparisons of scaled regression coefficient estimates (mean 0, range 2) for continuous variables along with evaluations of statistical significance. For categorical variables, parameter estimates were provided for a series of dummy variables representing all levels and statistical significance was determined for the difference between each level and the overall mean. Variables with significant parameter estimates were combined in an analysis of variance (ANOVA) to determine significant effects. A separate analysis of variance (ANOVA) was conducted using high and low hemoglobin categories (defined by the upper and lower 10th percentiles) to assess covariates potentially influencing values outside the normal range. Finally, piecewise linear regression models [McGee, 1970] with an inflection point were used to estimate linear relationships between infant cortisol and hemoglobin across the range of hemoglobin values. A nonlinear model (NLIN Procedure, SAS® Software, SAS Institute, Cary, North Carolina, USA) simultaneously estimated the inflection point and the corresponding slopes above and below the inflection point. P values less than or equal to 0.05 were considered statistically significant. Two-sided tests were used for all analyses.

RESULTS

A sample of 652 CBC's from three- to four-month-old rhesus infants born and reared in outdoor corrals over a three-year period (2002–2004) were analyzed. Characteristics of the dams and infants are shown in Table I. These variables were considered as possible hemoglobin predictors.

Figure 1 presents the hemoglobin distribution in the sample. The mean value was $12.2 \pm \text{SD } 0.8$ g/dL and the median value was 12.1 g/dL. Percentiles were used to identify three categories. The cutoff criterion for identifying a “low hemoglobin population” was a calculated value of less than 11.3 g/dL, which would fall within the lower 10th percentile of the distribution. Similarly, the cutoff criterion for identifying a “high hemoglobin” population was a calculated value of greater than 13.0 g/dL, which would fall within the upper 10th percentile of the distribution. Sixteen infants were classified as anemic based on hemoglobin values more than two standard deviations (SDs) below the mean, which is a commonly used criterion for identifying anemia in human newborns [Shao et al., 2012; Yip, 2000]. This criterion was 10.6 g/dL for this population. MCV is a second index that can indicate iron deficiency anemia. No infant had hemoglobin and MCV values that were both more than two SDs below the mean.

As anticipated, hemoglobin values were strongly associated in multiple regression with the number of red blood cells (RBC, $df=1,644$, $\beta=2.21$, $P<.0001$), the amount of hemoglobin per cell (MCH, $\beta=.36$, $df=1,644$, $P<.0001$), the size of the cells (MCV, $df=1,644$, $\beta=.06$, $P<.0001$), and the hemoglobin per unit volume (MCHC, $df=1,644$, $\beta=.08$, $P<.0001$). Plasma protein concentration, which reflects blood volume, and sample color, which reflects minor hemolysis, were not associated with the hemoglobin values. RBC and WBC numbers were not correlated, indicating that a higher general rate of hematopoiesis was not responsible for the higher RBC number and hemoglobin values.

Over the three-year period 499 dams contributed infants to the sample; about one-third contributed more than one infant. The distribution of hemoglobin values was similar in offspring from dams who contributed one, two or three infants to the sample (Table II).

Three sets of regressions were used to screen for relevant covariates (maternal, infant, and environmental factors) (Fig. 2). Variables that were significant within these groupings were combined for the final regression analysis.

Maternal reproductive history

The age of dams was highly related to all reproductive history variables, and was a significant predictor of infant hemoglobin (Fig. 2A). Graphing of these data showed that infant hemoglobin was stable up to a dam age of 10 years, but increased at the older ages. Dam parity (conceptions), number of previously reared infants, prior stillbirths/neonatal deaths were not significantly associated with infant hemoglobin in regression analysis that included age. None of the maternal variables differed by percentile category (low, normal, high hemoglobin).

It is important to note that relevant dam weight and hematology information were not available. Although colony records contained weight and CBC data, these measures were not taken in connection with BBA or at a standard time relative to the pregnancy producing the infants under study.

Infant sex, age, weight and plasma cortisol

Sex, age and weight were highly interrelated since males and older infants weighed more. Weight was the strongest predictor of hemoglobin (Fig. 2B). There was no effect of sex independent of weight. Hemoglobin decreased with infant age at which the CBC was obtained. Across the percentile comparison categories (low, normal, high hemoglobin), infant age, weight and sex did not differ by category.

Surprisingly, plasma cortisol, measured in the same blood sample used for the CBC, showed a strong association with hemoglobin (Fig. 3). This was the strongest predictive relationship in the study and it was not reduced by inclusion of other predictors in the ANOVA evaluating effects (Fig. 2D). Because of the relationship between cortisol and hemoglobin, further analysis was conducted to see if there was a threshold value at which the linear relationship with cortisol emerged. Piecewise regression demonstrated that cortisol and hemoglobin were associated at hemoglobin values greater than 11.7 mg/dL but not at lower values. Across the percentile comparison categories, the high hemoglobin group had significantly higher plasma cortisol than the low ($F=5.38$, $df=1.626$, $p<.01$) and normal ($F=9.67$, $df=1.626$, $P<.05$) groups.

Environmental variables

Birth location (corral cage of origin) was a highly significant predictor of hemoglobin values. Figure 2C shows that 15 of the 18 cages had mean hemoglobin values that did not differ from the overall average. However, infants from cage I and cage L had significantly lower hemoglobin values, and infants from cages B, J and R had significantly higher

hemoglobin values (see also Supplementary Table 1). The other two environmental variables, yearly breeding season and month of birth, did not influence infant hemoglobin (Fig. 2C).

DISCUSSION

This study found that maternal background (dam age), infant characteristics (infant weight and plasma cortisol) and environmental factors (corral cage of origin) contributed to the variability of hemoglobin in weanling rhesus.

Variables from the colony data base reflecting maternal reproductive history, such as parity and number of infants previously raised, were not found to be associated with infant hemoglobin. Colony records did not include information on gestation length and birth interval for animals housed outdoors. Older maternal age, which ranged from the age of reproductive maturity (3 years) to 19 years, was associated with greater infant hemoglobin. The maternal age variable could be an indirect representation of greater spacing between offspring at older ages; however, data analysis showed the number of conceptions per year of life did not decrease with age. It is also possible that the effect of maternal age was mediated by greater dam weight, which was not available in colony records. Information on dam iron status may also help clarify this situation. Offspring of dams with low iron stores (based on transferrin saturation) prior to conception were found to develop anemia as infants (based on MCV) [Lubach and Coe, 2006].

Infant weight was a predictor of hemoglobin independent of infant age and sex in effect screening. Heavier infants had higher hemoglobin values. More rapid postnatal growth is sometimes posited to deplete iron and lead to anemia. However, a positive, rather than a negative association has been seen in older children [Schneider et al., 2008; Yang et al., 2009]. Growth rate was not known in our study. Within this limited age range, hemoglobin was not found to change significantly with age but the direction of the association (greater age, lower hemoglobin) followed the expected maturational pattern [Jopling et al., 2009; Kling et al., 1996].

Among environmental variables, conception month and year did not influence hemoglobin but cage of origin had a strong effect. It is possible that conditions in the corral cages are strong determiners of infant hemoglobin when dietary iron is adequate. Because the cages are all in close proximity, of similar construction and maintained by the same husbandry protocols, it is difficult to suggest a physical origin for the cage effect. Although social dynamics may be relevant, the cages consist of stable social groups.

Genetics may be a factor that requires further examination. Heritability is known to be high for hematological endpoints. A number of genetic disorders result in anemia, as well as iron overload [Fleming and Ponka, 2012]. In human populations, genome-wide association studies (GWAS) have been undertaken with hemoglobin and other CBC measures to identify relevant associations [Benjamin et al., 2007; Ganesh et al., 2009; Kullo et al., 2010; Yang et al., 2007] and quantitative trait locus (QTL) studies of genetic influences on some CBC measures are also being undertaken in defined mouse populations in the Collaborative

Cross project [Kelada et al., 2012]. Rhesus monkey population genetic studies are currently underway at CNPRC and confirm that genetic diversity is attenuated under current housing and breeding protocols [Kanthaswamy et al., 2010]. A major factor contributing to genetic diversity is interbreeding of rhesus of Indian and Chinese origin [Kanthaswamy et al., 2009]. However, available data on the relative presence of Indian and Chinese origin monkeys in the different cages, the percent of inbreeding and the general population genetic heterogeneity, as well as the specific pathogen free (SPF) status, of the cages were not factors distinguishing corrals with highest as compared to lowest infant hemoglobin values. Differential prevalence of alleles with genes relevant to iron regulation in these cages might be uncovered by more specific genetic analyses.

The most striking and unexpected association was between infant cortisol and hemoglobin. The possible direction of causality cannot be confidently inferred from the information on hand. Anemia might be seen as a stressor that activates the adrenocortical system; however the relationship was not seen in the low hemoglobin range. Longstanding over-reactivity of the adrenocortical system prior to the BBA assessment may have altered erythropoiesis. It is well known that the adrenocortical hormones stimulate erythropoiesis [Morra et al., 1989] and they are used therapeutically for this purpose. A similar role of cortisol during embryonic-fetal erythropoiesis is suggested by the presence of glucocorticoid receptors on embryonic definitive erythroblasts that have extended proliferative ability [England et al., 2011]. If elevated cortisol in response to stress causes short-term hemoglobin elevation, this would be an important consideration whenever a post-capture blood sample is used to evaluate hematological function.

A possible physiological mechanism for a linear relationship between cortisol and hemoglobin in the higher range of values is spleen contraction mediated by adrenergic neurotransmitters of the autonomic nervous system in response to stress [Oka et al., 2007; Stewart and McKenzie, 2002]. Spleen contraction occurring in response to acute stressors such as hypoxia and strenuous exercise rapidly increases the circulating RBC count and hemoglobin content. The separation/relocation stress over the 2 hour period prior to blood sampling may have been effective in this regard. The average cortisol of the infants (78 µg/dL) is similar to levels seen by others in maternal-infant separation and relocation paradigms [Higley et al., 1992] but these higher cortisol values (2–3 times baseline) are rarely reported in infants under other conditions. Splenic contraction is considered an autonomic response; though less studied than adrenocortical activation, autonomic activation is seen in response to maternal-infant separation and relocation [Reite et al., 1978]. However no literature was found on spleen contraction in response to a psychological stressor.

This survey found that anemia is uncommon in our outdoor reared infants of early weaning age. This was not unexpected as anemia typically emerges in weanlings greater than six months of age. Twelve of 29 six- to seven-month old infants from a colony fed a diet with 230 ppm Fe were identified as anemic [Coe et al., 2012]. In an earlier study, 19% of three- to four-month-old indoor reared rhesus were identified with anemia [Bicknese et al., 1993]. MCV values indicative of anemia (69 fL) were reported as the average value in three-month old indoor mother-reared rhesus [Kriete et al., 1995]. The question arises as to whether the

three- to four-month-old infants in the present study with hemoglobin values at the low end of the distribution were anemic or in danger of developing anemia. No monkeys had anemia using the standard of both low hemoglobin and MCV two SD's below the mean, a standard of hemoglobin less than 11 g/dL and MCV less than 60 fL used by Coe and colleagues to classify anemia in six- to seven-month-old rhesus infants (Coe et al. 2012), or a criterion of hemoglobin less than 11 g/dL and MCV less than 65 fL previously used by us [Golub et al., 2009]. Other indices of iron deficiency such as transferrin saturation, serum ferritin and zinc protoporphyrin were not available. It is also possible that the spleen contraction mechanism would move anemic infants temporarily into a normal hemoglobin category. Unfortunately, it is not possible to ascertain the future anemic state of the infants surveyed here as they progress through the weaning process.

There are many studies of predictors of low hemoglobin as a marker of ID in human populations. Nutrition, cultural traditions concerning pregnancy and lactation, and socioeconomic status are predictors of infant ID. Infant sex, birth weight and postnatal weight gain are also predictors. Predictors of the range of "normal" hemoglobin values are not well studied. To date, genetics as a source of infant hemoglobin variability has received little attention. This study with environmental control uncovered the possibility of concomitant stress, social and genetic factors in predicting hemoglobin.

Although low hemoglobin as an indicator of anemia is a concern, high hemoglobin receives less attention [Yip, 2000]. Genetic iron storage disorders, hemochromatosis and beta thalassemia, lead to higher hemoglobin values which in turn have been associated with increased blood viscosity and poor pregnancy outcome. Environmental factors such as smoking and high altitude residence lead to elevated hemoglobin. Few other genetic and environmental sources of high circulating hemoglobin have been explored.

Using large populations of nonhuman primates maintained on standard diets with adequate iron, additional light may be shed on the genetic and environmental sources of hemoglobin variability and its consequences.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Population distribution of hemoglobin in a group of three- to four-month-old infants born over a three-year period at CNPRC in outdoor social housing. Whisker plot shows the mean and 95% confidence intervals (diamond), median (horizontal line in box), the first and third quartiles (top and bottom of box), 1.5 interquartile range + first and third quartiles (end of whiskers). N=652.

A. Maternal factors

B. Infant factors

C. Significant coefficients in regression equation including maternal, infant and environment variables

	F Ratio	Prob > F
Delivery age	5.567	0.0186*
Location	5.4656	<.0001*
Weight	5.9074	0.0154*
Cortisol	20.3326	<.0001*

D. Environmental factors

Figure 2.

Predictors of infant hemoglobin. N=650–652 due to absence of individual covariate values for individual animals. X-axes for graphs in A., B. and C. are scaled parameter estimates of regression coefficients from an effect screening model [SAS Institute Inc., 2012]. Solid vertical lines are boundaries for statistical significance of the estimates in the model. A. Maternal reproductive history parameters. B. Infant parameters. C. Environmental parameters. D. Analysis of variance for effects detected by effect screening and represented by standardized regression coefficients. N=650–652 due to absence of individual covariate values for individual animals.

Figure 3.
Scatterplot of the relationship between plasma cortisol and hemoglobin.

Table 1

Background Parameters. Data extracted from colony records and used as potential predictors in regression equations. N=650–652

Dam Reproductive History	mean $\pm$ SD	95% CI
Number of conceptions	3.59 $\pm$ 3.48 *	3.32 – 3.86
Prior biological rearing	2.18 $\pm$ 2.24	2.01 – 2.36
Prior live births	3.21 $\pm$ 3.19	2.97 – 3.46
Delivery age (years)	7.82 $\pm$ 3.76	7.53 – 8.10
First time rearing (Y/N)	168/483	
Prior neonatal death (Y/N)	48/603	
Infant Parameters		
Test age (days)	109.5 $\pm$ 9.9	108.7 – 110.3
Cortisol (μ g/mL)	78 $\pm$ 24	76 – 80
Test weight (kg)	1.01 $\pm$ 0.17	1.00 – 1.03
Sex (M/F)	271/381	
Environmental variables		
	values	range
Breeding season (year)	2002,2003,2004	
Corral cages (n=18)	A–R	
Corral cages sampled/year	15 $\pm$ 2 *	13–16 **
Month of birth (1–12, Jan–Dec)	3.8 $\pm$ 1.1	1–8
#infants/corral cage	36 $\pm$ 14 *	14–62 **

* mean $\pm$ SD,

** range

Table II

Distribution of hemoglobin values across low, normal and high categories for offspring of dams with different number of infants contributed to the sample.

Number of infants per dam	# dams	% low hgb<11.3	% normal hgb11.3-13.1	% high hgb>13.1
1	367	11	80	9
2	112	11	81	8
3	20	0	100	0