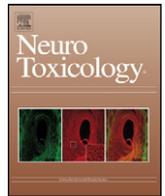




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## Delayed acquisition of neonatal reflexes in newborn primates receiving a thimerosal-containing Hepatitis B vaccine: Influence of gestational age and birth weight

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### ABSTRACT

This study examined whether acquisition of neonatal reflexes and sensorimotor skills in newborn rhesus macaques (*Macaca mulatta*) is influenced by receipt of the single neonatal dose of Hepatitis B (HB) vaccine containing the preservative thimerosal (Th). HB vaccine containing a standardized weight-adjusted Th dose was administered to male macaques within 24 h of birth ( $n = 13$ ). Unexposed animals received saline placebo ( $n = 4$ ) or no injection ( $n = 3$ ). Infants were raised identically and tested daily for acquisition of 9 survival, motor, and sensorimotor reflexes by a blinded observer. In exposed animals there was a significant delay in the acquisition of three survival reflexes: *root*, *snout* and *suck*, compared with unexposed animals. No neonatal responses were significantly delayed in unexposed animals compared with exposed. Gestational age (GA) and birth weight were not significantly correlated. Cox regression models were used to evaluate the main effects and interactions of exposure with birth weight and GA as independent predictors and time-invariant covariates. Significant main effects remained for exposure on *root* and *suck* when controlling for GA and birth weight such that exposed animals were relatively delayed in time-to-criterion. There was a significant effect of GA on *visual follow far* when controlling for exposure such that increasing GA was associated with shorter time-to-criterion. Interaction models indicated that while there were no main effects of GA or birth weight on *root*, *suck* or *snout* reflexes there were various interactions between exposure, GA, and birth weight such that inclusion of the relevant interaction terms significantly improved model fit. This, in turn, indicated important influences of birth weight and/or GA on the effect of exposure which, in general, operated in a way that lower birth weight and/or lower GA exacerbated the detrimental effect of vaccine exposure. This primate model provides a possible means of assessing adverse neurodevelopmental outcomes from neonatal Th-containing HB vaccine exposure, particularly in infants of lower GA or low birth weight. The mechanism of these effects and the requirements for Th is not known and requires further study.

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### 1. Introduction

The Hepatitis B (HB) vaccine was introduced into the US childhood immunization schedule in 1991 (Anon., 1991). This schedule recommended that all infants irrespective of gestational

age (GA) and birth weight born to HB-negative mothers be immunized with a HB vaccine within 12 h of birth (i.e. before hospital discharge (Anon., 1991, 1992)). We were unable to identify pre-clinical or prospective neurotoxicity studies that assessed the safety of this policy.

The two formulations of HB vaccine manufactured during the 1990s contained the preservative thimerosal (Th), an antibacterial and fungistatic agent composed of ethyl mercury and thiosalicylate. The HB vaccine contained 12.5  $\mu\text{g}$  ethyl mercury, given to neonates unadjusted for GA or birth weight. Following safety concerns, particularly in respect of the potential for neurotoxicity, a Congressionally mandated Food and Drug Administration (FDA)

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review in 1999 produced a recommendation for the reassessment of Th use in vaccines. By 2001 the majority of pediatric vaccines routinely recommended in the U.S. for children 6 years of age and under were produced without Th, with the exception of multi-dose inactivated influenza and meningococcal polysaccharide vaccines.

Since Th-containing vaccines, including the neonatal HB vaccine, continue to be used routinely in developing countries (Dórea et al., 2009), continued safety testing is important, particularly for premature and low birth weight neonates. Several formulations of HB vaccine currently used in other countries contain 25–50 µg ethyl mercury (Dórea et al., 2009). Based on the type of HB vaccine administered and the baby's birth weight there can be a 10-fold difference between the highest and lowest levels of mercury exposure to neonates (Dórea et al., 2009). In a U.S. study performed prior to the removal of Th from HB vaccines, blood mercury levels were significantly elevated in both pre-term and term infants post-HB vaccination (Stajich et al., 2000). While blood mercury levels are a poor reflection of body-burden of mercury, it is notable that these levels were higher in the pre-term infants when compared with term infants (Stajich et al., 2000). These findings suggest that newborns, especially pre-term infants, might have decreased ability to eliminate mercury since hepatic metallothionein and glutathione synthesis, both requirements for efficient mercury elimination, are not present in the neonate (Ono et al., 2002; Erden-Inal et al., 2002).

Here we examine, in a prospective, controlled, observer-blinded study, the development of neonatal reflexes in infant rhesus macaques after a single dose of Th-containing HB vaccine given within 24 h of birth, following the US childhood immunization schedule (1991–1999). The rhesus macaque is used in pre-clinical vaccine neurotoxicity testing and displays complex early neuro-behavioral and developmental processes that are well characterized (reviewed by Ruppenthal and Sackett, 1992). Neonatal motor reflexes, also referred to as 'survival' reflexes, are necessary for survival of macaques in the natural environment (Schneider and Suomi, 1992). If an infant primate is unable to suck, for example, they will not be able to obtain milk and are at risk of starvation. Neurobehavioral tests to assess early neonatal behavioral functioning are therefore commonly used to detect effects of post-natal events or interventions (Kroeker et al., 2007), such as exposure to organomercurials (Satoh et al., 1985). Macaques have also been used extensively in previous studies of methyl and ethyl mercury toxicokinetics and developmental neurotoxicity (Burbacher et al., 1990, 2005; Gunderson et al., 1986, 1988; Rice and Gilbert, 1982, 1990) making them a preferred model for addressing possible neurodevelopmental concerns regarding vaccine safety. Male primates were chosen because of the male preponderance of neurodevelopmental disorders and mounting evidence in support of a gender-selective neurotoxicity of organomercurials in both humans and animals (Rossi et al., 1997; Sakamoto et al., 1988; White, 2007; Branch, 2009; Malagutti et al., 2009; Gao et al., 2007). The objective of this study was to examine, in a primate model, whether administration of a single thimerosal-containing Hepatitis B vaccine at birth – the recommended pediatric protocol throughout the 1990s – resulted in delays in the acquisition of neonatal reflexes.

## 2. Materials and methods

### 2.1. Animal assurances

All procedures used in this research followed the guidelines of the Animal Welfare Act and the Guide for Care and Use of Laboratory Animals of the National Research Council. Research protocols were approved by the University of Pittsburgh/MWRI&F Institutional Animal Care and Use Committees (IACUCs).

### 2.2. Subjects

Twenty nursery-reared rhesus macaque infants served as study subjects at the Pittsburgh Development Center primate nursery. Pregnancies were produced by time-mated breeding. Fertile dams were selected based on their menses records and placed with a proven breeder for 4–5 days starting 2 days prior to expected ovulation. Mating was indicated by the presence of a seminal plug and GA confirmed by ultrasound at approximately 30 days. After delivery the health of infants was assessed by Simian APGAR, vital signs, and physical appearance. The birth weight and GA of the infant monkeys were within the normal range for this species; the average birth weight was 529 g (SD, 78.4 g; range, 394–688 g) and average GA was 168 days (SD, 5.51 days; range, 157–178 days).

### 2.3. Housing

Infants were separated from their mothers at birth and reared within a neonatal nursery according to the detailed protocols of Ruppenthal and Sackett (1992). Separation was necessary for this study as mothering precludes neonatal testing due to the distress caused to both the mother and the neonate when temporarily separated (Sackett et al., 2002; Suomi et al., 1983). The only way to rigorously test neonatal behavioral development is to remove the infant from its mother at birth (Ruppenthal and Sackett, 1992; Sackett et al., 2002). This protocol also ensures the survival of infants irrespective of birth weight or GA (Ruppenthal and Sackett, 2006). Environmental conditions were strictly controlled in the nursery to eliminate potential confounding factors such as diet or infant handling. Infants were similarly isolated, housed, and bottle-fed by hand in the nursery until achieving temperature regulation, typically 7–10 days from birth. For the first 3 days of life, vital statistics (respiration, heart rate, and temperature) were taken every 4 h. Infants that could self-regulate temperature during this 3-day period were moved out of their incubator and singly housed in a small nursery cage with a heating pad. If infants remained stable from Days 4 to 10, the heating pad was turned off. The cage had mesh walls on all sides to provide the infant with good visibility of his environment and also contained a cylindrical shaped hanging cloth surrogate suspended from the cage ceiling. Infants could see and hear each other but had no physical contact, both within and between peer groups for the duration of this study as per standard nursery procedures. Each cage also contained a formula feeder used to train infants to feed themselves. They received a standard infant baby formula (Enfamil, Mead Johnson and Co., IN). The appetite, attitude, activity level, hydration status, and stool quality of each animal were assessed by a nursery technician at each feeding. If infants were able to maintain health with the heating pad off and were self-feeding, their heating pads were removed by Day 14. Lights were on in the nursery from 0600 to 2000 h. Room temperature was maintained between 75 and 77 °F.

### 2.4. Study design

Animals were allocated to either the vaccinated (exposed) or saline/no injection (unexposed) groups on a semi-random basis in order to complete peer groups for later social testing (Ruppenthal and Sackett, 1992) such that each peer group contained animals from either the unexposed or exposed study groups. Once a new peer group was started, new animals were assigned to this group until it consisted of 3 or 4 infants, the ages of which were less than 4 weeks apart from their peers. Infants received either a single dose (0.5 ml) of Th-containing HB vaccine ( $n = 13$ ) or a saline injection ( $n = 4$ ) both administered i.m. in the thigh within 24 h of birth, or no injection ( $n = 3$ ).

## 2.5. Vaccine source, preparation and dosing

In July 1999, the CDC and American Academy of Pediatrics (AAP) recommended that thimerosal (Th) should be removed from pediatric vaccines, including all Hepatitis B (HB) formulations, which until that time contained 12.5 µg ethyl mercury per 0.5 ml dose. Therefore, in order to recreate the HB vaccine used in the 1990s, a Th-free preparation of this vaccine RECOMBIVAX HB<sup>®</sup> (Merck) was purchased and Th added as described below. The amount of Th added to the HB vaccines resulted in a concentration of 2.0 µg ethyl mercury per 0.5 ml dose. This represents a 6.25-fold reduction of ethyl mercury in macaque HB vaccines compared with those formulated for human use. This was necessary to adjust for the smaller size of the rhesus infants (Ruppenthal, 1989), thereby maintaining a similar ethyl mercury 'exposure' to human infants of ~2 µg kg/body weight. Purchased HB vaccines were pooled prior to Th addition. Th dosing and all QA/QC were performed at the University of Kentucky in the Environmental Research and Training Laboratory (ERTL). Stock Th (Sigma–Aldrich, St. Louis, MO) solutions were prepared such that a 50 µl dose added to the pooled vaccines would yield the desired ethyl mercury concentrations. Triplicate stock Th solutions and spiked vaccine solutions were digested in 5% nitric acid at 100 °C for 2 h and analyzed for ethyl mercury concentration using a Varian Vista Pro CCD Simultaneous Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) to verify that target concentrations were achieved. Matrix effects were evaluated and corrected for using an yttrium internal standard. Furthermore, second source curve verifiers and spike recoveries were in excess of 95%. Laboratory Control Samples (LCSs) consisting of three different dilutions of the

stock solutions bracketing the expected concentrations of the dosed vaccines were also prepared and analyzed alongside the dosed vaccines on a Nippon MA-2000 mercury analyzer. Recoveries on the LCSs were again in excess of 95%. The dosed HB vaccine contained ~2.0 µg ethyl mercury per 0.5 ml dose.

## 2.6. Neurodevelopmental testing

From birth, the development of neonatal and infant reflexes and perceptual and motor skills were assessed in all infants (Ruppenthal and Sackett, 1992). These were based on the Brazleton assessment scale, which was originally developed for human infants (Brazleton, 1978). Tests were performed daily and measured basic motor reflexes, visual and auditory orienting, muscle tone, and behavioral state as described below. Responses and scoring criteria are described in Table 1 and have been extensively published (Ruppenthal and Sackett, 1992; Schneider and Suomi, 1992; Kroeker et al., 2007; Chamove and Molinaro, 1978; Ruppenthal et al., 1991; Sackett et al., 2006). Neonatal assessments were performed by L.A.H. who was unaware of the study group assignment of each animal, the number of animals in each study group, and the number of study groups. L.A.H. underwent extensive training by G. Ruppenthal, one of the co-authors of the 'Research Protocol and Technician's Manual', the most exhaustive guide on the healthy development of nursery-raised infant macaques (Ruppenthal and Sackett, 1992). Training of L.A.H. was completed on non-study infants prior to the acquisition of data for this study and consisted of comparison of assessments of similarly aged infants collected by multiple trained testers. Reliability for nursery assessments by the trainee was achieved

**Table 1**  
Neonatal reflexes measured, rating categories, and criterion responses.

Test reflex	Rating category/definition	Criterion score
Rooting (left, right)	0 = no rooting 1 = partial rooting, does not move completely to object 2 = weak slow and/or intermittent move to object 3 = strong, quick vigorous move to object	3
Snouting	0 = none 1 = partial response, mouth not open 2 = weak, slow response (opens mouth) but sluggish 3 = strong, quick full response (opens mouth)	3
Sucking	0 = none 1 = partial response, mostly mouthing, little sucking 2 = weak sucking 3 = strong suck but biting first	3
Auditory startle	0 = none 1 = whole body jerk	1
Grasping (left, right; hands, feet)	0 = no grasp 1 = partial 2 = weak 3 = strong	3
Clasping	0 = no clasp 1 = loose clasp 2 = firm clasp 3 = climbs off	2
Auditory orient (left, right)	0 = none 1 = partial 2 = full orient	2
Visual orient (near, far)	0 = none 1 = head moves 2 = brief contact 3 = prolonged contact	3
Visual follow (left, right; near, far)	0 = no contact 1 = contact, no follow 2 = incomplete follow 3 = complete follow	3

when they obtained an 89% agreement with the trained testers on three consecutive randomized assessments. As part of standard protocol (Ruppenthal and Sackett, 1992), nursery testers who worked in the facility were routinely retested against each other at 3–6 months intervals.

All assessments of reflex acquisition were performed in a designated area under strictly controlled environmental conditions. Testing was always daily at a time intermediate between feedings when the neonate is least likely to be asleep or hungry (Ruppenthal and Sackett, 1992). For each test, the infant was removed from its cage, wrapped in a cloth diaper, and stimulated to perform the following ten behaviors: (i) four basic 'survival' reflexes including *root* (elicited by lightly brushing the cheek from ear to mouth), *snout* (elicited by brushing downward from the forehead between the eyes to the tip of the nose), *suck* (elicited by inserting a nipple into the mouth), and *auditory startle* (elicited by dropping a small metal object behind the infant's head); (ii) two motor reflexes including *grasp* (elicited by placing a finger in the palm of the hand and on the bottom of the foot), and *clasp* (elicited by placing the hands and feet around a cloth-covered cylinder held above the infant; and (iii) three sensorimotor behaviors including *auditory orienting* (elicited by lip smacking, a mother-to-infant communication gesture), *visual orienting and following* (elicited by positioning a small toy in front of the infant's face then moving the toy from left to right and right to left). The visual procedures were done with the toy positioned at either 12 or 24 in. in front of the infant representing both *near* and *far visual* tests, respectively. Although a total of 9 neonatal reflexes were measured, the inclusion of both hand/foot for *grasp* and *clasp* and near/far for visual procedures resulted in a total of 13 variables analyzed. The criterion for each measure was reached once the infant displayed the highest possible score (see Table 1).

### 2.7. Statistical analyses

Analyses were carried out using SPSS v. 17 (SPSS Inc., Chicago, IL). Analyses were based on the assessments of 20 animals from the day of birth to 14 days of age. The dependent variable in all survival analyses was hazard of time-to-criterion, measured for all neonatal reflexes and sensorimotor responses within this time frame. Right censoring of observations was applied when animals failed to reach criterion by Day 14. This was the required day of censoring since infants received further interventions on Day 14 which would have confounded the independent effects of the HB vaccine. Non-parametric Kaplan–Meier (K–M) log-rank estimation was used to estimate differences in age at criterion for each neonatal response between exposed and unexposed animals. Differences in survival curves associated with exposure status were tested using the log-rank test. The effect of GA and birth weight as independent predictors and time-invariant covariates was examined using Cox's regression.

The K–M log-rank test is a non-parametric method for comparing the survival experience of two or more groups, but it cannot be used to evaluate the effects of several variables on survival. The regression method introduced by Cox (Cox, 1972; proportional hazards regression analysis) is widely used when there is a need to investigate several variables at the same time. Variables entered into the model simultaneously generate output statistics which indicate the effect of each variable on outcome, while controlling for all other variables included in the model. These are referred to as 'main effects'. As in all regression modeling it is important to evaluate both the 'main effects' of variables and their possible interactions. Adding interaction terms allow exploration of the potentially differential effect of a variable across different exposure groups—asking the question of whether the effect of, in this case, either GA or birth weight, operates differently in exposed vs. unexposed animals.

Statistical significance was set at  $p \leq 0.05$  but variables approaching significance have also been noted where relevant. This is particularly important given the relatively low power of the study. Six variables included left-censored values for two unexposed animals who had reached criterion at first assessment; a score of 0.5 was allocated in each instance. For all regression models the  $\text{Exp}(\beta)$  variable is the main variable of interest indicating the predicted change in risk of meeting criterion (hazard) for a unit increase in the predictor.

When comparing models for goodness of fit (i.e. how well the particular model accounts for the outcome of interest) there are two relevant values. First, the likelihood-ratio test for the hypothesis that all parameters are 0 (no independent effects) is obtained by comparing the log-likelihood ( $x - 2; -2LL$ ) estimated for an Omnibus model in which all coefficients are 0, with the  $-2LL$  for the model that contains all the variables of interest. The lower the  $-2LL$ , the better the model. The difference between these models is also represented by the Chi-Square ( $\chi^2$ ) Change Statistic and its accompanying  $p$  value. After adding terms to the model, if the observed significance level of the 'Model Change  $\chi^2$ ' is small ( $\leq 0.05$ ) the null hypothesis, that all model coefficients are 0, can be rejected. None of the Cox Regression models violated the proportional hazards assumption which requires that the hazard ratio (hazard in exposed/hazard in unexposed) is constant over time.

## 3. Results

### 3.1. Infant health

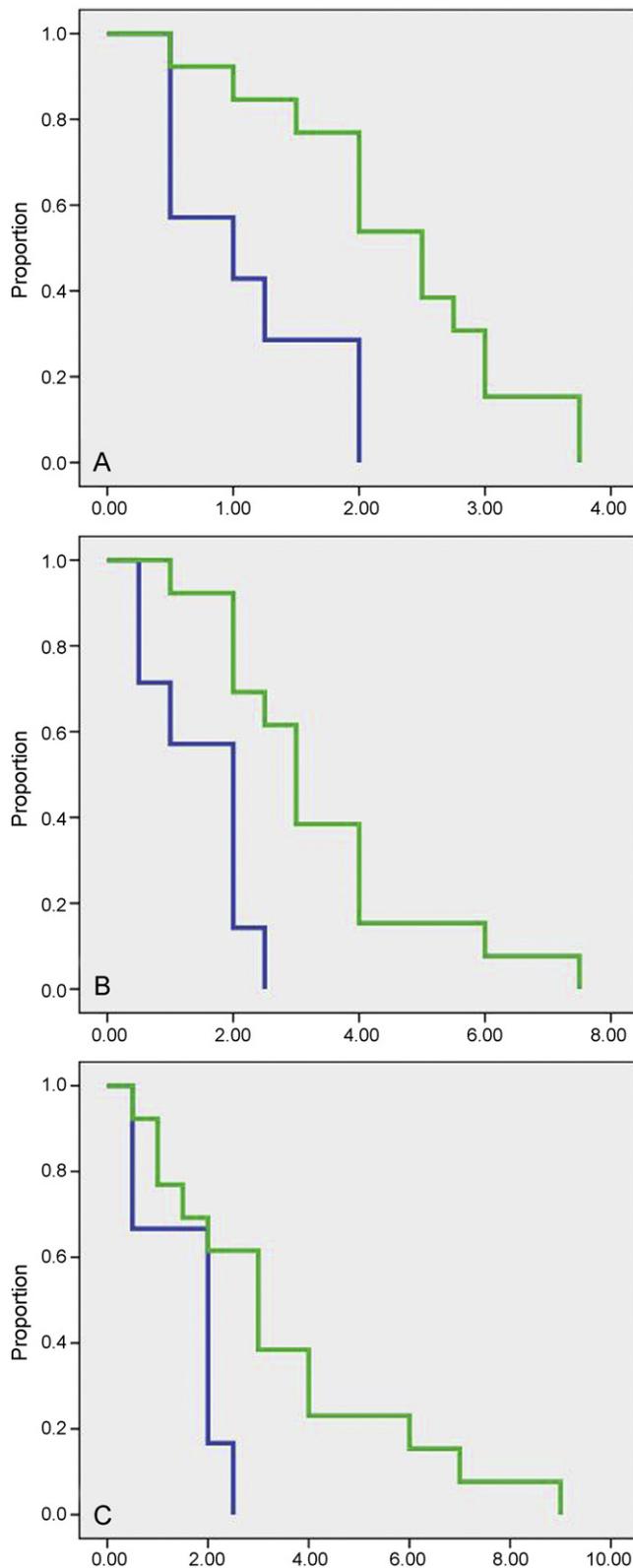
All infants remained healthy during the study testing period reaching all criteria for maintaining health including appetite, weight gain, and activity level, and achieved temperature regulation by Day 3. GA fell within normal range for all animals and was distributed normally in the sample, ranging from 160 to 178 in exposed animals (mean, 169.08; SD, 5.31) and from 157 to 176 in unexposed animals (mean, 167.57; SD, 6.16). Birth weight also fell within normal range for all animals. Distribution of birth weight was slightly skewed to the right in the sample, ranging from 394 to 688 in exposed animals (mean, 524.40; SD, 77.51; median, 502; IQR, 95.50) and from 449 to 643 in unexposed animals (mean, 538; SD, 85.54; median, 494; IQR, 171). There were no statistically significant differences between exposed and unexposed animals in either GA or birth weight ( $p > 0.5$ ) and no statistically significant correlation between GA and birth weight either for the sample as a whole, or for exposed vs. unexposed animals ( $p > 0.5$ ).

### 3.2. Neonatal development

Neonatal reflexes and sensorimotor responses were measured daily from birth until post-natal Day 14. Datasets from the two unexposed groups (with or without a saline injection) were combined when no differences were found for all measures ( $p > 0.5$ ). There was a significant delay in time-to-criterion for exposed vs. unexposed animals for three survival reflexes including *root* (Fig. 1A;  $p = 0.004$ ), *suck* (Fig. 1B;  $p = 0.002$ ) and *snout* (Fig. 1C;  $p = 0.03$ ) and approached significance for *startle* ( $p = 0.11$ ). The effect of exposure also approached significance for *grasp hand* ( $p = 0.07$ ), one of the motor reflexes. There were no reflexes for which the unexposed animals took significantly longer to reach criterion than the exposed animals (Table 2).

### 3.3. Modeling time-to-criterion: main effects models

Further evaluation of the potential impact of GA and/or birth weight on the association between exposure and outcome was



**Fig. 1.** The Kaplan–Meier (K–M) survival curve is a step-function used here to indicate the estimated proportion of animals having reached criterion at various time points from the start of the study period. K–M curves for neonatal reflexes in exposed (green) and unexposed (blue) rhesus macaque infants are presented: reflexes for time-to-criterion were measured daily from birth through Day 14. Significant differences were observed in the mean age (days) at reaching criterion for *root* (A;  $p = 0.004$ ), *suck* (B;  $p = 0.002$ ) and *snout* (C;  $p = 0.03$ ). The value on the y-axis represents the proportion of animals not reaching criterion by the time (in days) represented on the x-axis. Any point on the curve gives the proportion of infants still not having reached criterion at a particular time after the start of the

undertaken using Cox regression models. Before including potential covariates, unadjusted main effects of exposure were evaluated and confirmed for *root* ( $\text{Exp}(\beta) = 3.95$ ; 95% CI 1.29–12.05;  $p = 0.018$ ) and *suck* ( $\text{Exp}(\beta) = 4.58$ ; 95% CI 1.41–14.93;  $p = 0.011$ ). For *snout* the effect of exposure did not maintain its significance in the model ( $\text{Exp}(\beta) = 3.18$ ; 95% CI 0.952–10.615;  $p = 0.06$ ).

### 3.3.1. Exposure and gestational age (GA)

Cox regression modeling for main effects of exposure and GA together demonstrated a significant main effect of exposure for *root* ( $p = 0.009$ ) and *suck* ( $p = 0.015$ ) such that unexposed animals reached criterion more quickly. This approached statistical significance for *snout* ( $p = 0.055$ ). There was no main effect of GA for *root*, *suck* or *snout* ( $p > 0.05$ ). When GA was included in the model, a significant main effect of GA on *visual follow far* was observed ( $p = 0.013$ ) such that an increase in GA was associated with animals reaching criterion more quickly (Table 3). The unadjusted effect of GA on *visual follow far* was not statistically significant.

### 3.3.2. Exposure and birth weight

Cox regression modeling of exposure and birth weight demonstrated significant main effects that were almost identical to the models for exposure and GA described above. There were significant effects of exposure on *root* ( $p = 0.014$ ) and *suck* ( $p = 0.015$ ) such that unexposed animals reached criterion more quickly (Table 3). For *snout* the effect approached significance ( $p = 0.06$ ). There was no main effect of birth weight on any neonatal behavior (data not shown).

### 3.3.3. Modeling all three variables as main effects

Entering GA, birth weight, and exposure into the same model for each of the neonatal reflexes did not improve model fit and added nothing to the results, although exposure maintained its main effect on outcome for *root* ( $p = 0.009$ ) and *suck* ( $p = 0.02$ ) and approached significance for *snout* ( $p = 0.054$ ). GA maintained its independent effect on *visual follow far* ( $p = 0.017$ ).

## 3.4. Modeling time-to-criterion: interaction models

### 3.4.1. Gestational age (GA), birth weight and exposure status

To evaluate whether exposure was associated with different effects at different levels of GA and/or birth weight, and whether these covariates operated differently in exposed vs. unexposed infants, a number of interaction terms were included in the models for *root*, *suck* and *snout*.

For each neonatal reflex interaction terms were included together with main effects in a number of models as follows: Model (a): a three-way interaction between birth weight, exposure and GA; and 3 two-way interaction models: Model (b) between exposure and GA; Model (c) between exposure and birth weight; and Model (d) between birth weight and GA. Models (a) to (d) were compared in terms of log-likelihood statistics ( $-2LL$ ), with those showing a lower  $-2LL$ , with at least one statistically significant model term being optimal.

For the *root* reflex, the optimal interaction model was Model (d) which included the two-way interaction between GA and birth weight ( $p = 0.016$ ), together with the main effects of each. This had the lowest  $-2LL$  statistic of all *root* models described above (see Table 4 for all model parameters and relevant output) and compared favorably to the Omnibus Model. In the optimal *root*

observation period. For example, in A (*root*) 58% of the unexposed animals remained at Day 1 (meaning that 42% had reached criterion at this stage compared to around 9% of the exposed animals).

**Table 2**  
Neonatal reflexes: mean time-to-criterion with log-rank statistic and associated *p* values, for exposed (E) and unexposed (U) groups. Kaplan–Meier survival analysis was used to compare age in days at reaching neonatal basic motor reflexes and sensorimotor responses. The log-rank test identified statistically significant differences ( $p \leq 0.05$ ) for the achievement of these milestones between exposed and unexposed groups for three basic motor reflexes (*root*, *suck* and *snout*). There were no statistically significant differences between the groups in time-to-criterion for the remaining neonatal behaviors.

Reflex	Mean time-to-criterion (days)				Kaplan–Meier survival (log-rank)	
	Exposed ( $n = 13$ ) <sup>a</sup>		Unexposed ( $n = 7$ ) <sup>b</sup>		Chi-Square ( $\chi^2$ )	<i>p</i> -Value
	Mean	95% CI	Mean	95% CI		
<b>Survival reflexes</b>						
Root	2.33	1.80–2.85	1.11	0.61–1.61	8.19	0.004
Suck	3.38	2.42–4.35	1.50	0.90–2.10	9.52	0.002
Snout <sup>b</sup>	3.46	2.08–4.85	1.58	0.89–2.27	4.58	0.032
Auditory startle <sup>a</sup>	2.92	0.94–4.90	0.93	0.39–1.47	2.51	0.113
<b>Motor reflexes</b>						
Grasp hand	0.85	0.57–1.13	0.50	0.50–0.50	3.41	0.065
Grasp foot	0.77	0.56–0.98	0.7	0.26–1.24	0.36	0.956
Clasp hand	1.37	0.79–1.94	1.21	0.38–2.05	0.11	0.742
Clasp foot	1.65	0.97–2.34	1.29	0.59–1.98	0.69	0.407
<b>Sensorimotor reflexes</b>						
Auditory orient	3.21	1.89–3.77	2.83	0.65–5.78	0.32	0.570
Visual orient (Nr)	1.77	0.96–2.58	2.36	0.73–3.98	0.18	0.670
Visual orient (Far)	1.73	0.92–2.54	2.36	0.73–3.40	0.14	0.712
Visual following (Nr)	3.29	2.35–4.23	3.04	1.36–4.71	0.03	0.864
Visual following (Far)	2.91	1.48–4.33	3.68	1.87–5.49	0.88	0.348

CI, confidence interval; Nr, near.

<sup>a</sup> Censored at Day 14 (one infant in the exposed group did not reach criterion during the 14-day testing period for this study).

<sup>b</sup>  $n = 6$  for *snout* (scored as missing data for one infant).

**Table 3**  
Main effects models for exposure, gestational age (GA) and birth weight: significant predictors. For each neonatal behavior model, ‘–2LL’ gives the value of the model when all variables are included. Better models have lower values for –2LL. The change from the Omnibus Model (which assumes all effects are 0) to the specified model containing all variables, is represented by the  $\chi^2$  and its associated *p* value.  $p < 0.05$  indicates an improved model over the Omnibus. For model terms the  $\text{Exp}(\beta)$  or hazard ratio indicates the rise in risk of outcome (achievement of criterion) associated with a one unit rise in the predictor after controlling for all other terms in the model. Its associated value indicates the probability of an  $\text{Exp}(\beta)$  of this magnitude being generated by chance alone. For all analyses, exposure = 0 is the indicator variable. The exposure effect is therefore the effect of being unexposed. In other words for the *root* reflex, unexposure is associated with a 4.84 risk of meeting criterion for unexposed relative to an exposed animal. Other effects are of a rise in the predictor associated with a rise in risk hazard. Statistically significant *p* values for  $\text{Exp}(\beta)$  shown in bold font.

Reflex	Variables in model	Model –2LL	Change Omnibus to model		Model effects	$\text{Exp}(\beta)$	95% CI		<i>p</i> $\text{Exp}(\beta)$
			$\chi^2$	<i>p</i> ( $\chi^2$ )			Lower	Upper	
Root	Exposure	82.74	6.90	0.03	Exposure	4.841	1.481	15.821	<b>0.009</b>
	GA				Gestational age	1.046	0.966	1.133	0.264
	Exposure birth weight	83.67	5.97	0.05	Exposure	4.154	1.330	12.973	<b>0.014</b>
					Birth weight	1.002	0.996	1.007	0.555
	Exposure	82.74	6.90	0.07	Exposure	4.844	1.479	15.862	<b>0.009</b>
	GA				Gestational AGE	1.047	0.953	1.149	0.338
Suck	Birth weight				Birth weight	1.000	0.993	1.007	0.986
	Exposure	82.89	6.66	0.036	Exposure	4.411	1.337	14.559	<b>0.015</b>
	GA				Gestational age	0.979	0.902	1.062	0.609
	Exposure birth weight	81.89	7.65	0.022	Exposure	4.282	1.323	13.864	<b>0.015</b>
					Birth weight	0.996	0.990	1.003	0.282
	Exposure	81.75	7.80	0.05	Exposure	4.128	1.251	13.624	<b>0.020</b>
Visual follow far	GA				Gestational age	0.984	0.907	1.068	0.703
	Birth weight				Birth weight	0.996	0.990	1.003	0.304
	Exposure	77.70	8.31	0.016	Exposure	0.786	0.289	2.134	0.636
	GA				Gestational age	1.155	1.031	1.293	<b>0.013</b>
	Exposure birth weight	83.90	2.11	0.348	Exposure	0.689	0.264	1.800	0.447
					Birth weight	1.004	0.997	1.011	0.255
Snout	Exposure	76.97	9.04	0.03	Exposure	0.859	0.310	2.381	0.769
	GA				Gestational age	1.003	0.996	1.012	0.390
	Birth weight				Birth weight	1.158	1.027	1.306	<b>0.017</b>
	Exposure	77.61	3.84	0.15	Exposure	3.246	0.973	10.827	0.055
	GA				Gestational age	1.026	0.941	1.118	0.560
	Exposure birth weight	77.95	3.50	0.17	Exposure	3.171	0.949	10.600	0.061
Snout					Birth weight	1.000	0.994	1.006	0.930
	Exposure	77.33	4.12	0.25	Exposure	3.270	0.982	10.894	0.054
	GA				Gestational age	1.043	0.938	1.160	0.437
	Birth weight				Birth weight	0.998	0.991	1.005	0.601

**Table 4**

Best fit interaction models for root and suck reflexes. For each neonatal behavior model, '–2LL' gives the value of the model when all variables are included. Better models have a lower –2LL. The change from the Omnibus Model (which assumes all effects are 0) to the specified model containing all variables, is represented by the  $\chi^2$  and its associated  $p$  value.  $p < 0.05$  indicates an improved model over the Omnibus. For model terms the  $\text{Exp}(\beta)$  indicates the rise in risk of outcome (achievement of criterion) associated with a one unit rise in the predictor, after controlling for all other terms in the model. Its associated value indicates the probability of an  $\text{Exp}(\beta)$  of this magnitude being generated by chance alone. For all analyses, exposure = 0 is the indicator variable. While it is important to include main effects when modeling an interaction term, interpretation of any associated parameter estimates should be avoided when the interaction term is statistically significant. Parameter estimates for main effects are included here for reference and completeness. Statistically significant  $p$  values for  $\text{Exp}(\beta)$  shown in bold font.

Reflex	Variables in model	Model –2LL	Change Omnibus to model		Exp( $\beta$ )	95% CI		$p$ Exp( $\beta$ )
			$\chi^2$	$p$ ( $\chi^2$ )		Lower	Upper	
Root (Model d)	Exposure	77.14	12.22	0.016	6.669	1.886	23.575	<b>0.003</b>
	GA				2.689	1.074	6.734	<b>0.035</b>
	Birth weight				1.365	1.005	1.853	<b>0.046</b>
	GA $\times$ birth weight				0.998	0.996	1.000	<b>0.046</b>
Suck (Model a)	Exposure	76.78	12.76	0.012	0.004	0.000	1.746	0.075
	GA				0.936	0.856	1.025	0.153
	Birth weight				0.990	0.981	0.999	<b>0.038</b>
	Exposure $\times$ GA $\times$ birth weight				1.000	1.000	1.000	<b>0.024</b>
Suck (Model c)	Exposure	76.35	13.19	0.01	0.001	0.000	1.173	0.055
	GA				0.947	0.870	1.031	0.211
	Birth weight				0.990	0.980	0.999	<b>0.035</b>
	Exposure $\times$ birth weight				1.017	1.003	1.031	<b>0.020</b>
Snout (Model d)	Exposure	75.46	5.99	0.20	3.766	1.114	12.73	<b>0.033</b>
	GA				1.178	0.911	1.524	0.210
	Birth weight				1.697	0.796	3.616	0.171
	GA $\times$ birth weight				0.999	0.997	1.001	0.205

model, all terms were significant indicating that exposure ( $p = 0.003$ ), GA ( $p = 0.035$ ) and birth weight ( $p = 0.046$ ) are all significant factors in the acquisition of the 'root' reflex, when an interaction between GA and birth weight is allowed for.

For the *suck* reflex, two models fitted almost equally well: Model (a) a three-way interaction between GA, birth weight and exposure ( $p = 0.012$ ) and Model (c) a two-way interaction between exposure and birth weight ( $p = 0.01$ ). Both models compared favorably with, and were a significant improvement on, the Omnibus Model. The three-way model term of birth weight ( $p = 0.038$ ) and the three-way interaction between birth weight, GA and exposure ( $p = 0.024$ ) were statistically significant with exposure maintaining an effect that approached significance ( $p = 0.075$ ) once its interaction with birth weight and GA was controlled. For the two-way model (which included exposure and birth weight as the interaction term), both birth weight ( $p = 0.035$ ) and the interaction term ( $p = 0.02$ ) were statistically significant, with exposure maintaining an effect that approached significant ( $p = 0.055$ ).

For the *snout* reflex, the optimal interaction model was Model (d) which included the two-way interaction term between GA and birth weight together with the main effects of each. This had the lowest –2LL statistic of all *snout* models and compared favorably to the Omnibus Model (Table 4). In this model, although the interaction term was not in itself statistically significant, by controlling for the possible interaction between GA and birth weight, its inclusion strengthened the overall model and resulted in a significant effect of exposure ( $p = 0.033$ ). None of the remaining model terms GA, birth weight, and the interaction between GA and birth weight, were statistically significant ( $p > 0.05$ ). All interaction models reported here improved on model fit for models which included main effects alone.

In summary, the interaction models indicated that although there was no main effect of GA or birth weight on any variable other than *visual follow far*, there were various interactions between GA, birth weight and exposure, that, when controlled, added strength to the models being evaluated and indicated important influences of birth weight and/or GA on the effect of exposure. In general, the data

indicate that lower birth weight and/or lower GA exacerbates the effect of exposure, while increasing GA and/or birth weight mitigates the detrimental effect of exposure.

#### 4. Discussion

This study demonstrates that the acquisition of three neonatal survival reflexes, *root*, *suck* and *snout*, was significantly delayed in rhesus macaques receiving a single thimerosal (Th)-containing HB vaccine at birth. All infants remained healthy for the duration of the study suggesting that there were no health-related changes that may have affected the acquisition of reflexes. We sought, therefore, to examine what other variables might either account for, or influence, these observations. In general, as GA increased animals reached criterion earlier whereas animals of lower GA were relatively delayed. This effect was only significant when exposure was taken into account. For exposed animals, the effect of increasing GA was to mitigate the detrimental effect of exposure. Since there was no linear or additive relationship between GA and birth weight in this study, these observations cannot be readily accounted for by, for example, an effect of dose-of-exposure alone. It is plausible that while reflex acquisition *per se* is not influenced by GA alone, the brain of the less mature neonate may be more susceptible to neurotoxic injury manifesting as delayed reflex acquisition.

Although the basis for the effect of birth weight is not known, it is plausible that lower birth weight infants are more susceptible to what may be a dose-dependent toxicity of Th or some other HB vaccine constituent, such as aluminum. The effects on time-to-criterion appeared to be non-random: of the four survival reflexes, three were significantly negatively affected by exposure, while for *startle*, the fourth survival reflex, effects were similar but did not reach statistical significance. Interaction effects were observed with these same reflexes for both birth weight and GA. This interaction involved mitigation of the effect of exposure in a way that is biologically plausible, i.e. reduced time-to-criterion with increasing GA and birth weight.

Neurodevelopmental tests are used for both human and non-human primate neonates to study developmental status (Abrams

et al., 1995; Amiel-Tison et al., 1982; Karmel et al., 1998; Golub and Gershwin, 1984; Rogers, 1988) and these tests typically involve assessment of reflexes and orienting responses to visual and auditory stimuli as reported here. These test batteries are important in assessing the potential neurodevelopmental toxicity of chemicals and drugs (Laughlin et al., 1999; He et al., 2004; Stahlmann et al., 2007; Medoff-Cooper et al., 2009). They provide a broad-based evaluation of a range of nervous system functions at a period of life when learning and adaptation are particularly critical. Non-human primates are an especially appropriate test species because of their similarities to humans in complexity of brain function and prolonged intrauterine brain development (reviewed by Golub, 1990).

Our study design does not enable us to determine whether it is the vaccine *per se*, the exposure to Th, or a combination of both, that is causing the observed effects. None-the-less, the developing brain is considered the most vulnerable organ to mercury exposure (Grandjean and Perez, 2008), and experimental studies suggest that the brainstem – whose function is central to the reflexes described herein – may be one of the more sensitive targets (Sakamoto et al., 2001; Grandjean et al., 2004). Dietary methylmercury has been shown to accumulate in the brain of fish, resulting in histopathological damage, significantly reduced neural enzyme activity, and altered behavior (Berntssen et al., 2003). Pathological injury was observed to have started in the brainstem, extending to other areas of the brain at higher exposure levels. In a mouse model, exposure to mercury vapor resulted in a preferential accumulation of mercury in the brainstem, regardless of concentration used (Warfvinge, 1995). Similarly, after intramuscular injection, inorganic mercury accumulated in brainstem motor nuclei of mice (Arvidson, 1992). In clinical studies of mercury poisoning, exposure to organic mercury either pre- or post-natally resulted in brainstem defects in children (Amin-Zaki et al., 1978; Magos et al., 1985; Counter, 2003; Murata et al., 2004; Bernard et al., 2005). Since the acquisition of motor reflexes is controlled by the brainstem, it is possible that very early exposure to ethyl mercury may adversely affect emerging brainstem function (Wakefield et al., 2008). Brainstem injury may then disturb the development or functioning of higher structures (Geva and Feldman, 2008; Tanguay and Edwards, 1982; reviewed by McGinnis et al., *in press*).

We adjusted and standardized the Th concentration in order to give animals a clinically relevant exposure dose and allow meaningful comparisons. As in clinical practice, however, the final dose in terms of  $\mu\text{g}/\text{kg}$  body weight was dependent solely on the infants' birth weight. Stajich et al. (2000) examined blood mercury levels in US infants after receiving a single dose of HB vaccine and found the highest levels of mercury in pre-term infants suggesting that newborns, especially pre-term infants, may have decreased ability to eliminate mercury. Pichichero et al. (2008) also measured blood mercury in infants after receiving a single Th-containing HB vaccine at birth. Since blood samples were only collected once from each infant and at time points ranging from 12 h to 30 days post-exposure, it is not possible to draw any convincing conclusions from this study regarding the disposition of mercury in the blood of newborns after a single HB vaccine. Despite this, however, their data showed that in those infants for whom a blood sample was collected between 12 h to 2 days post-exposure, blood mercury levels varied greatly between subjects at each time point (Pichichero et al., 2008), suggesting that the ability to eliminate mercury varied among infants. Perhaps more importantly, the birth weight of infants ranged from 2.3 to 4.5 kg – almost a 2-fold difference – and yet data were not analyzed in respect of birth weight. Current pediatric immunization recommendations are for primary HB vaccination at birth (Anon., 1991, 1992) with no precautions for premature and/or low birth

weight babies (Anon., 1991). In fact the World Health Organization recommends that the birth-dose HB vaccine be given to pre-term infants but if their birth weight is  $<2000$  g, the vaccine dose at birth should not be counted towards the primary series, and three additional doses should be administered (WHO, 2004). Based upon the current findings in term, normal weight-range neonatal macaques, it may be that premature and/or low birth weight neonates are at increased risk of neurotoxicity. We have previously shown that very low birth weight and/or premature rhesus macaque infants display much longer delays in acquiring these same reflexes (Dettmer et al., 2007). It is notable that, within low birth weight animals, males have significantly delayed development for some reflexes relative to females, including the suck reflex (9). The acquisition of reflexes in infant primates of low birth weight and/or GA receiving a Th-containing HB vaccine at birth should be examined.

There have been several animal studies looking at the effects of thimerosal-containing vaccines (TCVs) and/or Th on neurodevelopment, behavior, immune function, and toxicology (Burbacher et al., 2005; Hornig et al., 2004; Havarinasab et al., 2005; Berman et al., 2008; Minami et al., 2009). Burbacher et al. (2005) examined the disposition and distribution of mercury in the brain of cynomolgus macaques administered methyl mercury or TCVs. In their study, cynomolgus macaques received TCVs and were sacrificed at various time points post-vaccination. While their data demonstrated that tissue distribution and clearance rates differed between methyl mercury and Th-exposed infants, the proportion of inorganic mercury in the brain was substantially higher for animals receiving TCVs (Burbacher et al., 2005). Once inorganic mercury has accessed the brain, its half-life is much longer than both ethyl and methyl mercury, and it has the potential to accumulate in cases of prolonged or repeated exposure (reviewed by Rooney, 2007). If, in our on-going investigations, Th is found to be driving the detrimental development effects, a dose–response study would be warranted. Our findings provide an important rationale for determining what factors in the HB vaccine may be responsible for these clinical observations. This should also include aluminum hydroxide which is used as an adjuvant in many vaccines, including the HB vaccine formulation used for this study. Studies are underway to examine this, and the consequences of repeated and/or additional vaccine exposures on the natural course of neurodevelopment.

## 5. Conclusions

In summary, this study provides preliminary evidence of abnormal early neurodevelopmental responses in male infant rhesus macaques receiving a single dose of Th-containing HB vaccine at birth and indicates that further investigation is merited. Birth weight and GA appear to be important variables that might predicate susceptibility. This study design was not able to determine whether it was the vaccine *per se*, the exposure to thimerosal, or a combination of both, that caused these effects. While primate testing forms an important part of pre-clinical safety assessment of vaccines intended for human use (Kennedy et al., 1997), the outcomes reported here are not included in the current CDC recommendations for Hepatitis B vaccine safety testing (Anon., 1982). A replication study in a larger cohort of infants is underway that extends these investigations to other areas of clinical concern such as emerging cognition, long-term learning and behavior, and neuroimaging studies of brain structure and function.

## Conflict of interest statement

Prior to 2005, CS and AJW acted as paid experts in MMR-related litigation on behalf of the court retained by plaintiff lawyers. LH

has a child who is a petitioner in the National Vaccine Injury Compensation Program. For this reason, LH was not involved in any data collection or statistical analyses to preclude the possibility of a perceived conflict of interest.

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