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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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This study examined whether acquisition of neonatal reflexes in newborn rhesus macaques was influenced by receipt of a single neonatal dose of hepatitis B vaccine containing the preservative thimerosal (Th). Hepatitis B vaccine containing a 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 tested daily for acquisition of nine survival, motor, and sensorimotor reflexes. In exposed animals there was a significant delay in the acquisition of *root*, *snout*, and *suck* reflexes, compared with unexposed animals. No neonatal responses were significantly delayed in unexposed animals. Gestational age (GA) and birth weight (BW) were not significantly correlated. Cox regression models were used to evaluate main effects and interactions of exposure with BW and GA as independent predictors and time-invariant covariates. Significant main effects remained for exposure on *root* and *suck* when controlling for GA and BW, such that exposed animals were relatively delayed in time-to-criterion. Interaction models indicated there were various interactions between exposure, GA, and BW and that inclusion of the relevant interaction terms significantly improved model fit. This, in turn, indicated that lower BW and/or lower GA exacerbated the adverse effects following vaccine exposure. This primate model provides a possible means of assessing adverse neurodevelopmental outcomes from neonatal Th-containing hepatitis B vaccine exposure, particularly in infants of lower GA or BW. The mechanisms underlying these effects and the requirements for Th requires further study.

The hepatitis B (HB) vaccine was introduced into the U.S. childhood immunization schedule in 1991 (Centers for Disease Control, 1991). This schedule recommended that all infants irrespective of gestational age (GA) and birth weight (BW) born to HB-negative mothers be immunized with a HB vaccine within 12 h of birth, that is, before hospital discharge (American Academy of Pediatrics, 1992; Centers for Disease Control, 1991). It should be noted that it was not possible to identify preclinical 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 ethylmercury and thiosalicylate. The HB vaccine contained 12.5 μg ethylmercury and was given to neonates unadjusted for GA or BW. Following safety concerns, particularly with respect to potential neurotoxicity, a congressionally mandated Food and Drug Administration (FDA) 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 United States for children 6 yr of age and under were produced without Th, with the exception of multidose inactivated influenza vaccines.

Since Th-containing vaccines, including the neonatal HB vaccine, continue to be used routinely in developing countries (Dorea 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 ethylmercury (Dorea et al., 2009). Based on the type of HB vaccine administered and the neonate's BW, there may be a 10-fold difference between the highest and lowest levels of mercury (Hg) exposure to neonates (Dorea et al., 2009). In a U.S. study performed prior to the removal of Th from HB vaccines, blood Hg levels were significantly elevated in both preterm and term infants post HB vaccination (Stajich et al., 2000). While blood Hg levels are a poor biomarker for body burden of this metal,

it is noteworthy that these levels were higher in preterm compared to term infants (Stajich et al., 2000). These findings suggest that newborns, especially preterm infants, might have decreased ability to eliminate Hg (Graeter & Mortesen, 1996; Kern & Jones, 2006) since hepatic metallothionein and glutathione synthesis, both requirements for efficient Hg elimination, are not present in the neonate (Ono et al., 2001; Erden-Inal et al., 2002).

The purpose of this study was to conduct a prospective, controlled, observer-blinded study involving the development of neonatal reflexes in infant rhesus macaques administered a single dose of Th-containing HB vaccine within 24 h of birth, following the U.S. childhood immunization schedule (1991–1999). The rhesus macaque is commonly used in preclinical vaccine neurovirulence testing and displays complex early neurobehavioral and developmental processes that are well characterized (Ruppenthal & Sackett, 1992). Neonatal motor reflexes, also referred to as "survival" reflexes, are necessary for survival of macaques in the natural environment (Schneider & Suomi, 1992). If an infant primate is not able to suck, the animal is not able to obtain milk and is at risk for starvation. Neurobehavioral tests to assess early neonatal behavioral functioning are therefore commonly used to detect effects of postnatal events or interventions (Kroeker et al., 2007), including exposure to organomercurials (Satoh et al., 1985). Macaques have also been used extensively in previous studies on methyl- and ethylmercury toxicokinetics and developmental neurotoxicity (Burbacher et al., 1990, 2005; Gunderson et al., 1986, 1988; Rice & Gilbert, 1982, 1990), making them a preferred model for addressing potential neurodevelopmental concerns regarding vaccine safety. Male primates were selected because of the preponderance of neurodevelopmental disorders in males and mounting evidence in support of a gender-selective neurotoxicity of organomercurials in both humans and animals (Rossi et al., 1997; Sakamoto et al., 1998; White, 2007; Branch, 2009; Malagutti et al., 2009; Gao et al., 2007).

MATERIALS AND METHODS

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 and MWRI&F Institutional Animal Care and Use Committees (IACUC).

Animals

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 d, starting 2 d prior to expected ovulation. Mating was indicated by the presence of a seminal plug and GA confirmed by ultrasound at approximately 30 d. After delivery the health of infants was assessed by Simian APGAR, vital signs, and physical appearance. The BW and GA of the infant monkeys were within the normal range for this species; the average BW was 529 g (SD 78.4 g; range, 394–688 g) and average GA was 168 d (SD 5.51 d; range, 157–178 d).

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 produced to both mother and neonate when temporarily separated (Sackett et al., 2002; Suomi et al., 1983). The only currently known way to rigorously test neonatal behavioral development is to remove the infant from its mother at birth (Ruppenthal & Sackett, 1992; Sackett et al., 2002). This protocol also ensures the survival of infants irrespective of BW or GA (Ruppenthal & 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 d from birth. For the first 3 d of life, vital statistics (respiration, heart rate, and temperature) were taken every 4 h. Infants that could self-regulate temperature during this 3-d period were moved out of their incubator and housed singly in a small nursery cage with a heating pad. If infants remained stable from d 4–10, 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. Animals 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 d 14. Lights were on in the nursery from 0600 h to 2000 h. Room temperature was maintained between 24 and 25°C.

Study Design

Animals were allocated to either the vaccinated (exposed) or saline/no injection (unexposed) groups on a semirandom basis in order to complete peer groups for later social testing (Ruppenthal & 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 wk 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 im in the thigh within 24 h of birth, or no injection ($n = 3$).

Vaccine Source, Preparation, and Dosing

In July 1999, the Centers for Disease Control (CDC) and American Academy of Pediatrics (AAP) recommended that Th should be removed from pediatric vaccines, including all hepatitis B (HB) formulations, which until that time contained 12.5 μg ethylmercury/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 (Merck) was purchased and Th was added as described below. The amount of Th added to the HB vaccines resulted in a concentration of approximately 2 μg ethylmercury/0.5-ml dose. This represents a 6.25-fold reduction of ethylmercury 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 an ethylmercury "exposure" similar to that of human infants. Purchased HB vaccines were pooled prior to Th addition. Th dosing and all quality assurance/quality control (QA/QC) procedures 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 ethylmercury 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 (LCS) 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 LCS were again in excess of 95%. The dosed HB vaccine contained approximately 2 μg ethylmercury/0.5-ml dose.

Neurodevelopmental Testing

From birth, the development of neonatal and infant reflexes and perceptual and motor skills were assessed in all infants (Ruppenthal & Sackett, 1992). These were based on the Brazleton assessment scale, which was originally developed for human infants (Brazelton, 1978). Tests were performed daily and measured basic motor reflexes, visual and auditory orienting, muscle tone, and behavioral state as described later. Responses and scoring criteria are described in Table 1 and extensively published (Ruppenthal & Sackett, 1992; Schneider & Suomi, 1992; Kroeker et al., 2007; Chamove & 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 from 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 & Sackett, 1992). Training of L.A.H. was completed on nonstudy 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 when they obtained an 89% agreement with the trained testers on 3 consecutive randomized assessments. As part of standard protocol (Ruppenthal & Sackett, 1992), nursery testers who worked in the facility were routinely retested against each other at 3- to 6-mo 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 & Sackett, 1992). For each test, the infant was removed from its cage, wrapped in a cloth diaper, and

stimulated to perform the following 10 behaviors: (i) 4 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) 2 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) 3 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 conducted with the toy positioned at either 12 or 24 inches in front of the infant representing both *near* and *far* visual tests, respectively. Although 9 neonatal reflexes were measured, the inclusion of both hand and foot for *grasp* and *clasp* and *near* and *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).

Statistical Analyses

Analyses were carried out using SPSS v. 17 (SPSS, Inc., Chicago). Analyses were based

TABLE 1. Neonatal Reflexes Measured, Rating Categories, and Criterion Responses

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

on the assessments of 20 animals from the day of birth to 14 d 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 d 14. This was the required day of censoring, since infants received further interventions on d 14 that would have confounded the independent effects of the HB vaccine. Nonparametric 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 BW as independent predictors and time-invariant covariates was examined using Cox's regression.

The K-M log-rank test is a nonparametric 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 (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 that 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 allows exploration of the potentially differential effect of a variable across different exposure groups—asking whether the effect of, in this case, either GA or BW, operates differently in exposed versus unexposed animals.

Statistical significance was set at $p \leq .05$ 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 2 unexposed animals who had reached criterion at first assessment; a score of 0.5 was allocated in each instance. For all regression

models the $\exp(\beta)$ 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 (times −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 is the model. The difference between these models is also represented by the chi-square (χ^2) change statistic and its accompanying p value. After adding terms to the model, if the observed significance level of the "model change χ^2" is small ($p \leq .05$) 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.

RESULTS

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 d 3. Gestational age (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 (BW) also fell within normal range for all animals. Distribution of BW was numerically skewed to the right in the sample, ranging from 394 to 688 in exposed animals (mean 524.40, SD 77.51; median 502, interquartile range [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 BW and no statistically significant correlation between GA and BW either for the sample as a whole, or for exposed versus unexposed animals.

Neonatal Development

Neonatal reflexes and sensorimotor responses were measured daily from birth until postnatal d 14. Data sets from the two unexposed groups (with or without a saline injection) were combined when no statistical differences were found for all measures. There was a significant delay in time-to-criterion for exposed versus unexposed animals for three survival reflexes including *root* (Figure 1A), *suck* (Figure 1B), and *snout* (Figure 1C) but not significant for *startle*. The effect of exposure approached significance for grasp hand, 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).

Modeling Time-to-Criterion: Main Effects Models

Further evaluation of the potential impact of GA and/or BW on the association between exposure and outcome was undertaken using Cox regression models. Before including potential covariates, unadjusted main effects of exposure were evaluated and confirmed for significant for *root* ($\exp(\beta)$ = 3.95, 95% CI 1.29–12.05) and *suck* ($\exp(\beta)$ = 4.58; 95% CI 1.41–14.93). For *snout* the effect of exposure did not maintain its significance in the model ($\exp(\beta)$ = 3.18; 95% CI 0.952–10.615).

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* and *suck* such that unexposed animals reached criterion more quickly. This was not significant for *snout*. There were no marked main effects of GA for *root*, *suck*, or *snout*. When GA was included in the model, a significant main effect of GA on *visual follow far* was observed such that an

increase in GA was associated with animals reaching criterion more quickly (Table 3). The unadjusted effect of GA on *visual follow fa*r was not statistically significant.

Exposure and birth weight Cox regression modeling of exposure and BW demonstrated significant main effects that were almost identical to the models for exposure and GA described earlier. There were significant effects of exposure on *root* and *suck* such that unexposed animals reached criterion more quickly (Table 3). For *snout* the effect was not significant. There were no marked main effects of BW on any neonatal behavior (data not shown).

Modeling all three variables as main effects Entering GA, BW, 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 significant effect on outcome for *root* and *suck* but not for *snout*. GA maintained its significant independent effect on *visual follow far*.

Modeling Time-to-Criterion: Interaction Models

Gestational age, birth weight, and exposure status To evaluate whether exposure was associated with different effects at different levels of GA and/or BW, and whether these covariates operated differently in exposed versus 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 BW, exposure, and GA; and three two-way interaction models: model (b), between exposure and GA; model (c), between exposure and BW; and model (d), between BW 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 significant interaction between GA and
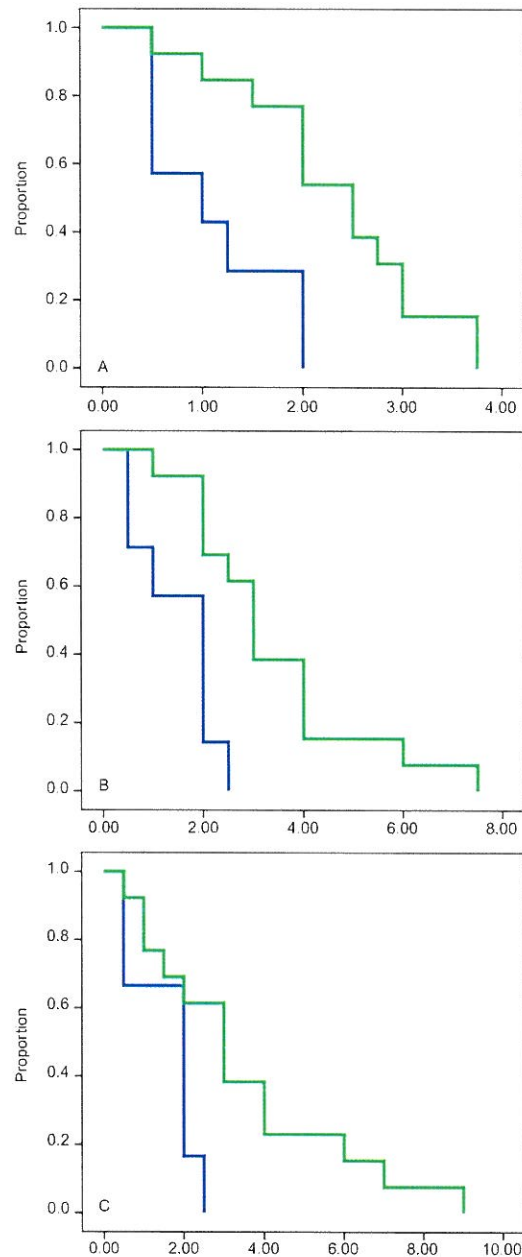
FIGURE 1. The Kaplan–Meier (K-M) survival curve is a step function used here to indicate the estimated proportion of animals having reached criterion at various time points from the start of the study period. K-M curves for neonatal reflexes in exposed (green) and unexposed (blue) rhesus macaque infants are presented; reflexes for time-to-criterion were measured daily from birth through d 14. Significant differences were observed in the mean age (days) at reaching criterion for *root* (A; $p = .004$), *suck* (B; $p = .002$), and *snout* (C; $p = .03$). 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 observation period. For example, in A (*root*), 58% of the unexposed animals remained at d 1 (meaning that 42% had reached criterion at this stage, compared to around 9% of the exposed animals).

BW, together with the main effects of each. This had the lowest $-2LL$ statistic of all *root* models described earlier (Table 4 for all model parameters and relevant output) and compared favorably to the omnibus model. In the optimal *root* model, all terms were significant, indicating that exposure, GA, and BW are all significant factors in the acquisition of the *root*

TABLE 2. Neonatal Reflexes: Mean Time-to-Criterion With Log-Rank Statistic and Associated *p* Values, for Exposed (E) and Unexposed (U) Groups

	Mean time-to-criterion (days)				Kaplan–Meier survival (log rank)	
	Exposed (n = 13)**		Unexposed (n = 7)*			
Reflex	Mean	95% CI	Mean	95% CI	Chi square (χ^2)	*p* Value
Survival reflexes						
Root	2.33	1.80–2.85	1.11	0.61–1.61	8.19	.004
Suck	3.38	2.42–4.35	1.50	0.90–2.10	9.52	.002
Snout*	3.46	2.08–4.85	1.58	0.89–2.27	4.58	.032
Auditory startle**	2.92	0.94–4.90	0.93	0.39–1.47	2.51	.113
Motor reflexes						
Grasp hand	0.85	0.57–1.13	0.50	0.50–0.50	3.41	.065
Grasp foot	0.77	0.56–0.98	0.7	0.26–1.24	0.36	.956
Clasp hand	1.37	0.79–1.94	1.21	0.38–2.05	0.11	.742
Clasp foot	1.65	0.97–2.34	1.29	0.59–1.98	0.69	.407
Sensorimotor reflexes						
Auditory orient	3.21	1.89–3.77	2.83	0.65–5.78	0.32	.570
Visual orient (Nr)	1.77	0.96–2.58	2.36	0.73–3.98	0.18	.670
Visual orient (Far)	1.73	0.92–2.54	2.36	0.73–3.40	0.14	.712
Visual following (Nr)	3.29	2.35–4.23	3.04	1.36–4.71	0.03	.864
Visual following (Far)	2.91	1.48–4.33	3.68	1.87–5.49	0.88	.348

Note. 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 ($p \leq .05$) for the achievement of these milestones between exposed and unexposed groups for three survival reflexes (*root*, *suck*, and *snout*). There were no statistically significant differences between the groups in time-to-criterion for the remaining neonatal behaviors. CI, confidence interval; Nr, near. Asterisk indicates $n = 6$ for *snout* (scored as missing data for one infant); double asterisk, censored at d 14 (one infant in the exposed group did not reach criterion during the 14-d testing period for this study).

reflex, when an interaction between GA and BW is allowed for.

For the *suck* reflex, two models fitted almost equally well: model (a), a three-way significant interaction between GA, BW and exposure, and model (c), a two-way significant interaction between exposure and BW. Both models compared favorably with, and were a significant improvement on, the omnibus model. The three-way model term of birth weight and the three-way interaction between BW, GA, and exposure were statistically significant with exposure maintaining an effect that approached significance once its interaction with BW and GA was controlled. For the two-way model (which included exposure and BW as the interaction term), both birth weight and the interaction term were statistically significant, with exposure maintaining an effect that approached significant.

For the *snout* reflex, the optimal interaction model was model (d), which included the two-way interaction term between GA and BW 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 BW, its inclusion strengthened the overall model and resulted in a significant effect of exposure. None of the remaining model terms, GA, BW, and the interaction between GA and BW, were statistically significant. All interaction models reported here improved on model fit for models that included main effects alone.

DISCUSSION

This study demonstrated that the acquisition of three neonatal survival reflexes, *root*, *suck*, and *snout*, was significantly delayed in rhesus macaques receiving a single 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

TABLE 3. Main Effects Models for Exposure, Gestational Age (GA), and Birth Weight: Significant Predictors

Reflex	Variables in model	Model −2LL	Change omnibus to model χ^2	$p\ (\chi^2)$	Model effects	Exp (β)	95% CI Lower	Upper	p Exp (β)
Root	Exposure	82.74	6.90	.03	Exposure	4.841	1.481	15.821	**.009**
	GA				Gestational age	1.046	0.966	1.133	.264
	Exposure	83.67	5.97	.05	Exposure	4.154	1.330	12.973	**.014**
	Birth weight				Birth weight	1.002	0.996	1.007	.555
	Exposure	82.74	6.90	.07	Exposure	4.844	1.479	15.862	**.009**
	GA				Gestational age	1.047	0.953	1.149	.338
	Birth weight				Birth weight	1.000	0.993	1.007	.986
Suck	Exposure	82.89	6.66	.036	Exposure	4.411	1.337	14.559	**.015**
	GA				Gestational age	0.979	0.902	1.062	.609
	Exposure	81.89	7.65	.022	Exposure	4.282	1.323	13.864	**.015**
	Birth weight				Birth weight	0.996	0.990	1.003	.282
	Exposure	81.75	7.80	.05	Exposure	4.128	1.251	13.624	**.020**
	GA				Gestational age	0.984	0.907	1.068	.703
	Birth weight				Birth weight	0.996	0.990	1.003	.304
Visual follow far	Exposure	77.70	8.31	.016	Exposure	0.786	0.289	2.134	.636
	GA				Gestational age	1.155	1.031	1.293	**.013**
	Exposure	83.90	2.11	.348	Exposure	0.689	0.264	1.800	.447
	Birth weight				Birth weight	1.004	0.997	1.011	.255
	Exposure	76.97	9.04	.03	Exposure	0.859	0.310	2.381	.769
	GA				Gestational age	1.003	0.996	1.012	.390
	Birth weight				Birth weight	1.158	1.027	1.306	**.017**
Snout	Exposure	77.61	3.84	.15	Exposure	3.246	0.973	10.827	.055
	GA				Gestational age	1.026	0.941	1.118	.560
	Exposure	77.95	3.50	.17	Exposure	3.171	0.949	10.600	.061
	Birth weight				Birth weight	1.000	0.994	1.006	.930
	Exposure	77.33	4.12	.25	Exposure	3.270	0.982	10.894	.054
	GA				Gestational age	1.043	0.938	1.160	.437
	Birth weight				Birth weight	0.998	0.991	1.005	.601

Note. 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 χ^2 and its associated p value; $p < .05$ indicates an improved model over the omnibus. For model terms the exp(β) 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 exp(β) 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. Bolded text indicates statistically signifcant values.

of reflexes. In general, as GA increased, animals reached criteria earlier, whereas animals of lower GA were relatively delayed in attaining criteria. 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 BW in this study, these observations cannot be readily accounted for by 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 (Makri et al., 2004; Kern & Jones, 2006). Although the basis for the effect of BW is not known, it is plausible that lower BW 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 nonrandom: 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

TABLE 4. Best Fit Interaction Models for Root and Suck Reflexes

Reflex	Variables in model	Model −2LL	Change omnibus to model		Exp (β)	95% CI		p Exp(β)
			χ^2	$p\,(\chi^2)$		Lower	Upper	
Root	Exposure	77.14	12.22	.016	6.669	1.886	23.575	**.003**
	GA				2.689	1.074	6.734	**.035**
	Birth weight				1.365	1.005	1.853	**.046**
	GA × Birth weight				0.998	0.996	1.000	**.046**
Suck (Model a)	Exposure	76.78	12.76	.012	0.004	0.000	1.746	.075
	GA				0.936	0.856	1.025	.153
	Birth weight				0.990	0.981	0.999	**.038**
	Exposure × GA × Birth weight				1.000	1.000	1.000	**.024**
Suck (Model b)	Exposure	76.35	13.19	.01	0.001	0.000	1.173	.055
	GA				0.947	0.870	1.031	.211
	Birth weight				0.990	0.980	0.999	**.035**
	Exposure × Birth weight				1.017	1.003	1.031	**.020**
Snout	Exposure	75.46	5.99	.20	3.766	1.114	12.730	**.033**
	GA				1.178	0.911	1.524	.210
	Birth weight				1.697	0.796	3.616	.171
	GA × Birth weight				0.999	0.997	1.001	.205

Note. 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 χ^2 and its associated p value; $p < .05$ indicates an improved model over the omnibus. For model terms the exp(β) 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 exp(β) 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. Bolded text indicates statistically signifcant values.

statistical significance. Interaction effects were observed with these same reflexes for both BW 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 BW.

Neurodevelopmental tests are used for both human and nonhuman primate neonates to study developmental status (Abrams et al., 1995; Amiel-Tison et al., 1982; Karmel et al., 1998; Golub & Gershwin, 1984; Rogers, 1988), and these tests typically involve assessment of reflexes and orienting responses to visual and auditory stimuli. 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). These tests provide a broad-based evaluation of a range of nervous system functions at a period of life when learning and adaptation are particularly critical. Nonhuman primates are an especially appropriate test species because of their similarities to humans

in complexity of brain function and prolonged intrauterine brain development (Golub 1990).

The present 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 was responsible for the observed effects. However, the developing brain is considered most susceptible to Hg exposure (Grandjean & 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 (MeHg) was shown to accumulate in the brain of fish, resulting in histopathological damage, significantly reduced neural enzyme activity, and altered behavior (Ratcliffe et al., 1996; Sweet & Zelikoff, 2001; 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 Hg vapor resulted in a preferential accumulation of Hg in the brainstem, regardless of concentration used

(Warfvinge 1995). Similarly, after intramuscular injection, inorganic Hg accumulated in brainstem motor nuclei of mice (Arvidson 1992). In clinical studies of Hg poisoning, exposure to organic Hg either pre- or postnatally resulted in brainstem defects in children (Amin-Zaki et al., 1978; Magos et al., 1985; Counter et al., 2002; 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 early exposure to ethylmercury may adversely affect emerging brainstem function (Wakefield et al., 2008). Brainstem injury may then disturb the development or functioning of higher structures (Geva & Feldman, 2008; Tanguay & Edwards, 1982; McGinnis et al., 2010).

Th concentration was adjusted and standardized 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 micrograms per kilogram body weight was dependent solely on the infants' birth weight. Stajich et al. (2000) examined blood Hg levels in U.S. infants after receiving a single dose of HB vaccine and found the highest levels of Hg in preterm infants, suggesting that newborns, especially preterm infants, may have decreased ability to eliminate Hg. Pichichero et al. (2008) also measured blood Hg 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 d postexposure, it is not possible to draw any convincing conclusions from this study regarding the disposition of Hg in blood of newborns after a single HB vaccine. Despite this, however, data showed that in those infants for whom a blood sample was collected between 12 h and 2 d postexposure, blood Hg levels varied greatly between subjects at each time point (Pichichero et al., 2008), suggesting that the ability to eliminate Hg varied among infants. Perhaps more importantly, the BW of infants ranged from 2.3 to 4.5 kg—almost a twofold difference, and yet data were not analyzed with respect to BW. Current pediatric immunization recommendations are for primary HB vaccination at birth

(Centers for Disease Control, 1991; American Academy of Pediatrics, 1992) with no precautions for premature and/or low-BW babies (Centers for Disease Control, 1991). In fact, the World Health Organization recommends that the birth-dose HB vaccine be given to preterm infants but if their BW is <2000 g, the vaccine dose at birth should not be counted toward the primary series, and three additional doses should be administered (World Health Organization, 2004). Based upon the current findings in term, normal weight-range neonatal macaques, it may be that premature and/or low-BW neonates are at increased risk of neurotoxicity. Dettmer et al. (2007) previously showed that low-BW and/or premature rhesus macaque infants display much longer delays in acquiring these same reflexes. It is notable that, within low-BW animals, males have significantly delayed development for some reflexes relative to females, including the *suck* reflex (Kroeker et al., 2007). The acquisition of reflexes in infant primates of low BW and/or GA receiving a Th-containing HB vaccine at birth needs to be examined.

There have been several animal studies examining the effects of Th-containing vaccines (TCV) 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 Hg in the brain of cynomolgus macaques administered MeHg or TCV. In their study, cynomolgus macaques received TCV and were sacrificed at various time points post vaccination. While data demonstrated that tissue distribution and clearance rates differed between MeHg- and Th-exposed infants, the proportion of inorganic Hg in the brain was substantially higher for animals receiving TCV (Burbacher et al., 2005). Once inorganic Hg has accessed the brain, its half-life is much longer than for both ethyl- and methylmercury, and it has the potential to accumulate in cases of prolonged or repeated exposure (Rooney 2007). If, in our ongoing 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.

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. The interaction models indicated that lower BW and/or lower GA exacerbates the effect of exposure, while increasing GA and/or BW mitigates the detrimental effect of exposure. Birth weight and GA therefore 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 Th, or a combination of both that produced 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 (American Academy of Pediatrics, 1992). 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.

REFERENCES

Abrams, S. M., Field, T., Scafidi, F., and Prodromidis, M. 1995. Newborns of depressed mothers. *Infant Mental Health J.* 16:233–239.

American Academy of Pediatrics. 1992. Committee on Infectious Diseases universal hepatitis B immunization. *Pediatrics* 89:795–800.

Amiel-Tison, C., Barrier, G., Shnider, S. M., Levinson, G., Hughes, S. C., and Stefani, S. J. 1982. A new neurologic and adaptive capacity scoring system for evaluating obstetric medications in full-term newborns. *Anesthesiology* 56:340–350.

Amin-Zaki, L., Majeed, M.A., Clarkson, T. W., and Greenwood, M. R. 1978. Methylmercury poisoning in Iraqi children: Clinical observations over two years. *Br. Med. J.* 1:613–616.

Arvidson, B. 1992. Accumulation of inorganic mercury in lower motoneurons of mice. *Neurotoxicology* 13:277–280.

Berman, R. F., Pessah, I. N., Mouton, P. R., Mav, D., and Harry, J. 2008. Low-level neonatal thimerosal exposure: Further evaluation of altered neurotoxic potential in SJL mice. *Toxicol. Sci.* 101:294–309.

Bernard, S., Enayati, A., Redwood, L., Roger, H.,and Binstock, T. 2005. Autism: A novel form of mercury poisoning. *Med. Hypoth.* 56:462–471.

Berntssen, M. H., Aatland, A., and Handy, R. D. 2003. Chronic dietary mercury exposure causes oxidative stress, brain lesions, and altered behaviour in Atlantic salmon (*Salmo salar*) parr. *Aquat. Toxicol.* 65:55–72.

Branch, D. R. 2009. Gender-selective toxicity of thimerosal. *Exp. Toxicol. Pathol.* 61: 133–136.

Brazelton, T. B. 1978. The Brazelton Neonatal Behavior Assessment Scale: Introduction. *Monogr. Soc. Res. Child Dev.* 43:1–13.

Burbacher, T. M., Sackett, G. P., and Mottet, N. K. 1990. Methylmercury effects on the social behavior of Macaca fascicularis infants. *Neurotoxicol. Teratol.* 12:65–71.

Burbacher, T. M., Shen, D. D., Liberato, N., Grant, K. S., Cernichiari, E., and Clarkson, T. 2005. Comparison of blood and brain mercury levels in infant monkeys exposed to methylmercury or vaccines containing thimerosal. *Environ. Health Perspect.* 113:1015–1021.

Centers for Disease Control. 1991. Hepatitis B: A comprehensive strategy for eliminating transmission in the United States through universal childhood vaccination: Recommendations of the Immunization Practices Advisory Committee (ACIP). *Morbid. Mortal. Weekly Rep. (MMWR)* 40:1–19.

Chamove, A. S., and Molinaro, T. J. 1978. Monkey retardate learning analysis. *J. Mental Defic. Res.* 22:37–48.

Counter, S. A. 2003. Neurophysiological anomalies in brainstem responses of mercury-exposed children of Andean gold miners. *J. Occup. Environ. Med.* 45:87–95.

Counter, S. A., Buchanan, L. H., Ortega, F., and Laurell, G. 2002. Elevated blood mercury and neuro-otological observations in children of the Ecuadorian gold mines. *J. Toxicol. Environ. Health A* 65:149–163.

Cox, D. R. 1972. Regression models and life tables (with discussion). *J. R. Stat. Soc. Ser. B* 34:187–220.

Dettmer, A. M., Houser, L. A., Ruppenthal, G. C., Capuano, S., and Hewitson, L. 2007. Growth and developmental outcomes of three high-risk infant rhesus macaques (*Macaca mulatta*). *Am. J. Primatol.* 69:503–518.

Dorea, J. G., Marques, R. C., and Brandao, K. G. 2009. Neonate exposure to thimerosal mercury from hepatitis B vaccines. *Am. J. Perinatol.* 26:523–527.

Erden-Inal, M., Sunal, E., and Kanbak, G. 2002. Age-related changes in the glutathione redox system. *Cell Biochem. Funct.* 20:61–66.

Gao, Y., Yan, C. H., Tian, Y., Wang, Y., Xie, H. F., Zhou, X., Yu, X. D., Yu, X. G., Tong, S., Zhou, Q. X., and Shen, X. M. 2007. Prenatal exposure to mercury and neurobehavioral development of neonates in Zhoushan City, China. *Environ. Res.* 105:390–399.

Geva, R., and Feldman, R. 2008. A neurobiological model for the effects of early brainstem functioning on the development of behavior and emotion regulation in infants: Implications for prenatal and perinatal risk. *J. Child Psychol. Psychiat.* 49:1031–1041.

Golub, M. S. 1990. Use of monkey neonatal neurobehavioral test batteries in safety testing protocols. *Neurotoxicol. Teratol.* 12:537–541.

Golub, M. S., and Gershwin, M. E. 1984. Standardized neonatal assessment in the rhesus monkey. In *Research in perinatal medicine*, eds. P. Nathanielsz and J. Parer, pp. 56–87. Ithaca, NY: Perinatology Press.

Grandjean, P., Murata, K., Budtz-Jørgensen, E., and Weihe. P. 2004. *The brainstem as a target of developmental methylmercury toxicity.* 7th International Conference on Mercury as a Global Pollutant, at Ljubljana, Slovenia.

Grandjean, P., and Perez, M. 2008. Development neurotoxicity: implications of methylmercury research. *Int. J. Environ. Health* 2:417–428.

Graeter, L. J., and Mortensen, M. E. 1996. Kids are different: Developmental variability in toxicology. *Toxicology* 111:15–20.

Gunderson, V. M., Grant-Webster, K. S., Burbacher, T. M.,and Mottet, N. K. 1988. Visual recognition memory deficits in methylmercury-exposed *Macaca fascicularis* infants. *Neurotoxicol. Teratol.* 10:373–379.

Gunderson, V. M., Grant, K. S., Burbacher, T. M., Fagan, J. F., and Mottet, N. K. 1986. The effect of low-level prenatal methylmercury exposure on visual recognition memory in infant crab-eating macaques. *Child Dev.* 57:1076–1083.

Havarinasab, S., Haggqvist, B., Bjorn, E., Pollard, K. M., and Hultman, P. 2005. Immunosuppressive and autoimmune effects of thimerosal in mice. *Toxicol. Appl. Pharmacol.* 204:109–121.

He, N., Bai, J., Champoux, M., Suomi, S. J., and Lidow, M. S. 2004. Neurobehavioral deficits in neonatal rhesus monkeys exposed to cocaine *in utero*. *Neurotoxicol. Teratol.* 26:13–21.

Hornig, M., Chian, D., and Lipkin, W. I. 2004. Neurotoxic effects of postnatal thimerosal are mouse strain dependent. *Mol. Psychiat.* 9:833–845.

Karmel, B. Z., Gardner, J. M., and Freedland, R. L. 1998. Neonatal neurobehavioral assessment Bayley I and II scores of CNS-injured and cocaine-exposed infants. *Ann. NY Acad. Sci.* 846:391–395.

Kennedy, R. C., Shearer, M. H., and Hildebrand, W. 1997. Nonhuman primate models to evaluate vaccine safety and immunogenicity. *Vaccine* 15:903–908.

Kern, J. K., and Jones, A. M. 2006. Evidence of toxicity, oxidative stress, and neuronal insult in autism. *J. Toxicol. Environ. Health B* 9:485–499.

Kroeker, R., Sackett, G., and Reynolds, J. 2007. Statistical methods for describing developmental milestones with censored data: Effects of birth weight status and sex in neonatal pigtailed macaques. *Am. J. Primatol.* 69:1313–1324.

Laughlin, N. K., Lasky, R. E., Giles, N. L., and Luck, M. L. 1999. Lead effects on neurobehavioral development in the neonatal rhesus monkey (*Macaca mulatta*). *Neurotoxicol. Teratol.* 21:627–638.

Magos, L., Brown, A. W., Sparrow, S., Bailey, E., Snowden, R. T., and Skipp, W. R. 1985. The comparative toxicology of ethyl- and methyl-mercury. *Arch. Toxicol.* 57:260–267.

Makri, A., Goveia, M., Balbus, J., and Parkin, R. 2004. Children's susceptibility to chemicals: A review by developmental stage. *J. Toxicol. Environ. Health B* 7:417–435.

Malagutti, K. S., da Silva, A. P., Braga, H. C., Mitozo, P. A., Soares dos Santos, A. R., Dafre, A. L., de Bem, A. F., and Farina, M. 2009. 17ß-Estradiol decreases methylmercury-induced neurotoxicity in male mice. *Environ. Toxicol. Pharmacol.* 27:293–297.

McGinnis, W. R., Miller, V. M., Audhya, T., and Edelson, S. 2010. *Neurotoxic brainstem impairment as proposed threshold event in autistic regression.* In: Autism: Oxidative Stress, Inflammation and Immune Abnormalities, ed. A. Chauhan, V. Chauhan and T. Brown, pp. 153–176, Boca Raton: CRC Press, Taylor and Francis Group.

Medoff-Cooper, B., Shults, J., and Kaplan, J. 2009. Sucking behavior of preterm neonates as a predictor of developmental outcomes. *J. Dev. Behav. Pediatr.* 30:16–22.

Minami, T., Miyata, E., Sakamoto, Y., Yamazaki, H., and Ichida, S. 2009. Induction of metallothionein in mouse cerebellum and cerebrum with low-dose thimerosal injection. *Cell Biol. Toxicol.* 26:143–152.

Murata, K., Weihe, P., Budtz-Jorgensen, E., Jorgensen, P. J., and Grandjean, P. 2004. Delayed brainstem auditory evoked potential latencies in 14-year-old children exposed to methylmercury. *J. Pediatr.* 144:177–183.

Ono, H., Sakamoto, A., and Sakura, N. 2001. Plasma total glutathione concentrations in healthy pediatric and adult subjects. *Clin. Chim. Acta* 312:227–229.

Pichichero, M. E., Gentile, A., Giglio, N., Umido, V., Clarkson, T., Cernichiari, E., Zareba, G., Gotelli, C., Gotelli, M., Yan, L., and Treanor, J. 2008. Mercury levels in newborns and infants after receipt of thimerosal-containing vaccines. *Pediatrics* 121:208–214.

Ratcliffe, H. E., Swanson, G. M., and Fischer, L. J. 1996. Human exposure to mercury: A critical assessment of the evidence of adverse health effects. *J. Toxicol. Environ. Health* 49:221–270.

Rice, D. C., and Gilbert. S. G. 1982. Early chronic low-level methylmercury poisoning in monkeys impairs spatial vision. *Science* 216:759–761.

Rice, D. C., and Gilbert, S. G. 1990. Effects of developmental exposure to methyl mercury on spatial and temporal visual function in monkeys. *Toxicol. Appl. Pharmacol.* 102:151–163.

Rogers, W. R. 1988. Behavioral testing. In *Nonhuman primates in perinatal research*, eds. Y. Brans and T. Keuhl, pp. 411–420. New York: John Wiley and Sons.

Rooney, J. P. 2007. The role of thiols, dithiols, nutritional factors and interacting ligands in the toxicology of mercury. *Toxicology* 234:145–156.

Rossi, A. D., Ahlbom, E., Ogren, S. O., Nicotera, P., and Ceccatelli, S. 1997. Prenatal exposure to methylmercury alters locomotor activity of male but not female rats. *Exp. Brain Res.* 117:428–436.

Ruppenthal, G. C. 1989. *Weight gain and intake requirements in nursery-reared macaques.* Paper read at Proc. 4th Annual Dr. Scholl Conference on the Nutrition of Captive Wild

Animals at Lincoln Park Zoological Society, Chicago.

Ruppenthal, G. C., and Sackett, G. P. 1992. *Research protocol and technician's manual*, 2nd ed. Seattle: Infant Primate Research Laboratory, University of Washington.

Ruppenthal, G. C., Walker, G. C., and Sackett, G. P. 1991. Rearing infant monkeys (*Macaca nemestrina*) in pairs produces deficient social development compared with rearing in single cages. *Am. J. Primatol.* 25:103–113.

Ruppenthal, G., and Sackett, G. P. 2006. Nursery care of at-risk nonhuman primates. In *Nursery rearing of nonhuman primates in the 21st century*, eds. G. C. Ruppenthal, G. P. Sackett, and K. Elias, pp. 371–390. Chicago: University of Chicago.

Sackett, G. P., Ruppenthal, G. C., and Davis, A. E. 2002. Survival, growth, health, and reproduction following nursery rearing compared with mother rearing in pigtailed monkeys (*Macaca nemestrina*). *Am. J. Primatol.* 56:165–183.

Sackett, G., Ruppenthal, G., Hewitson, L., Simerly, C., and Schatten, G. 2006. Neonatal behavior and infant cognitive development in rhesus macaques produced by assisted reproductive technologies. *Dev. Psychobiol.* 48:243–265.

Sakamoto, M., Nakano, A., and Akagi, H. 2001. Declining Minamata male birth ratio associated with increased male fetal death due to heavy methylmercury pollution. *Environ. Res.* 87:92–98.

Sakamoto, M., Wakabayashi, K., Kakita, A., Takahashi, H., Adachi, T., and Nakano, A. 1998. Widespread neuronal degeneration in rats following oral administration of methylmercury during the postnatal developing phase: A model of fetal-type minamata disease. *Brain Res.* 784:351–354.

Satoh, H., Yasuda, N., and Shimai, S. 1985. Development of reflexes in neonatal mice prenatally exposed to methylmercury and selenite. *Toxicol. Lett.* 25:199–203.

Schneider, M. L., and Suomi, S. J. 1992. Neurobehavioral assessment in rhesus monkey neonates (*Macaca mulatta*): Developmental changes, behavioral stability, and early experience. *Infant Behav. Dev.* 15:155–177.

Stahlmann, N., Hartel, C., Knopp, A., Gehring, B., Kiecksee, H., and Thyen, U. 2007. Predictive value of neurodevelopmental assessment versus evaluation of general movements for motor outcome in preterm infants with birth weights <1500 g. *Neuropediatrics* 38:91–99.

Stajich, G. V., Lopez, G. P., Harry, S. W., and Sexson, W. R. 2000. Iatrogenic exposure to mercury after hepatitis B vaccination in preterm infants. *J. Pediatr.* 136:679–681.

Suomi, S.J., Mineka, S., and DeLizio, R. D. 1983. Short- and long-term effects of repetitive mother-infant separations on social development in rhesus monkeys. *Dev. Psychol.* 19:770–786.

Sweet, L. I., and Zelikoff, J. T. 2001. Toxicology and immunotoxicology of mercury: A comparative review in fish and humans. *J. Toxicol. Environ. Health B* 4:161–205.

Tanguay, P. E., and Edwards, R. M. 1982. Electrophysiological studies of autism: The whisper of the bang. *J. Autism Dev. Disord.* 12:177–184.

Wakefield, A.J., Stott, C., Lopresti, B. J., Tomko, J., Houser, L., Sackett, G., and Hewitson, L. 2008. *Pediatric vaccines influence primate behavior, and brain stem volume and opioid ligand binding*. 4th Annual International Meeting for Autism Research (IMFAR). London, UK.

Warfvinge, K. 1995. Mercury distribution in the mouse brain after mercury vapour exposure. *Int. J. Exp. Pathol.* 76:29–35.

White, J. F. 2007. Thimerosal: Chemistry, toxicology and effects on biological systems. In *Autism research challenges*, ed. L. B. Zhao, pp. 137–167. Nova Science.

World Health Organization. 2004. Hepatitis B Vaccines: WHO Position paper. *Weekly Epidemiol. Rec. (WER)* 79:263–274.