

Lack of Association Between Rh Status, Rh Immune Globulin in Pregnancy and Autism

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Received 4 October 2006; Accepted 4 April 2007

Though causes of autism are considered largely genetic, considerable concern remains that exposure to Rh immune globulin (RhIg), which until 2001 in the United States contained the preservative thimerosal, can cause autism. To determine whether mothers of children with autism are more likely to be Rh negative (Rh⁻) or to have received RhIg preserved with thimerosal, which is 49.6% ethyl mercury, we surveyed families of children with an autism spectrum disorder (ASD) ascertained through a University-based autism clinic considered free of ascertainment biases related to type of autism or severity. Between 2004 and 2006, 305 mothers of 321 children with an ASD agreed to participate in a telephone interview. Analysis of complete records including the blood group status and RhIg exposure of 214 families showed that Rh⁻ status is no higher in mothers of children with autism than in the general population, exposure to antepartum RhIg, preserved with

thimerosal is no higher for children with autism and pregnancies are no more likely to be Rh incompatible. This was also true for autism subgroups defined by behavioral phenotype, gender, IQ, regressive onset, head circumference, dysmorphology, birth status, essential, or complex phenotype. These findings support the consensus that exposure to ethylmercury in thimerosal is not the cause of the increased prevalence of autism. These data are important not only for parents in this country but also for the international health community where thimerosal continues to be used to preserve multi-dose vials which in turn makes vaccines affordable. © 2007 Wiley-Liss, Inc.

Key words: autism; Rh; Rh immune globulin; thimerosal; RhoGam

How to cite this article: Miles JH, Takahashi TN. 2007. Lack of association between Rh status, Rh immune globulin in pregnancy and autism. *Am J Med Genet Part A* 143A:1397–1407.

INTRODUCTION

Autism is a complex neurodevelopmental disorder diagnosed wholly on the basis of children's social interactions, communication, imaginative play and stereotypic activities [American Psychiatric Association, 1994]. First described in the 1940s by Kanner [1943] and Asperger [1944], autism remained within the purview of psychiatry until the mid-1980s. The last two decades have witnessed a remarkable swell in interest and concern about autism by professionals, families and government agencies. Families have come together to form autism support groups, such as The Autism Society of America (<http://www.autism-society.org>) and Autism Speaks (<http://www.autismspeaks.org>) which have publicized autism and lobbied Congress for increased appropriations for research and treatment. Autism-dedicated clinics have sprung up in most states. Though geneticists anticipate autism will be the first behavioral/psychiatric disorder for which major genes will be identified, fierce debate persists about

whether environmental toxins can also cause this childhood disorder.

As autism has become a common developmental diagnosis there have been adjustments in diagnostic criteria; what were previously considered discrete disorders are now referred to as the autism spectrum disorders (ASDs), ranging from classical autistic disorder to milder Asperger syndrome and a variety of behavioral subtypes subsumed by the term pervasive developmental disorders (PDD). Over the same period, the prevalence of the diagnosis has increased significantly in all populations where

Grant sponsor: Johnson and Johnson Company; Grant sponsor: Leda J. Sears Trust.

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DOI 10.1002/ajmg.a.31846

it has been tracked. Prior to 1990, most studies estimated the prevalence for autism at four to five per 10,000 [Jorde et al., 1990; Fombonne, 2001]. During the 1990s, studies of preschool children in Japan, England, and Sweden reported autism prevalence rates of 21 to 31 per 10,000 [Honda et al., 1996; Arvidsson et al., 1997; Baird et al., 2000]. An important epidemiologic study from the United Kingdom utilizing specialized visiting nurses who monitored child health and development reported a prevalence rate of 16.8 per 10,000 for autism and 63 per 10,000 for all the PDDs in children younger than 5 years [Chakrabarti and Fombonne, 2001]. Those rates agree with a CDC case—finding study in Brick Township, New Jersey, updated results from Chakrabarti and Fombonne [2005], and two population based prevalence studies conducted by the CDC in the United States [Centers for Disease Control and Prevention, 2006]. Based on the increase in reported cases, many have worried that the actual incidence of autism is increasing; perhaps in epidemic proportions. This has led to concerns that some new environmental exposures or toxins could account for the increase.

The primary focus of this concern has been early childhood immunizations which are given from birth through age 5 years. The addition of five pediatric vaccines (hepatitis B, varicella, haemophilis b, pneumococcal, and influenza) to the immunization schedule in the last 25 years [Pickering, 2003], is temporally consistent with the increasing prevalence of autism [Luyster et al., 2005; Werner and Dawson, 2005]. Possible ill effects of the various attenuated pathogens themselves have been looked for without consistent evidence of a causal relationship [Peltola et al., 1998; Wakefield et al., 1998; Wakefield, 1999; DeWilde et al., 2001; Kaye et al., 2001; Hviid et al., 2003; Honda et al., 2005; Rutter, 2005]. Most notably has been the controversy over whether the MMR immunization somehow causes retention of measles viral particles in the children's intestines which either release toxins or set up an immune response that could be directed to the brain. Others have expressed concern that the sheer volume of injected antigens could be deleterious.

Equal attention has been directed toward thimerosal which has been used as a preservative in multi-dose vaccine vials since the early 1930s. Thimerosal, which is 49.6% ethylmercury by weight, is considered a potential suspect because of the increased ethylmercury exposure per child from the added immunizations, the temporal correspondence to the increase in autism diagnoses and recognition of the teratogenic effects of methylmercury following the large industrial spill in Minimata Bay, Japan [Harada, 1995]. Acting on these concerns, the FDA conducted a review of thimerosal in childhood vaccines and though they found no evidence of harm, noted that some low birth weight infants could

have been exposed to cumulative levels of mercury during the first 6 months of life which exceed EPA recommended guidelines for safe intake of methylmercury [Ball et al., 2001]. As a precautionary measure the Public Health Service, the Health Resources and Services Administration, the Center for Disease Control and Prevention and the American Academy of Pediatrics issued a joint statement, urging vaccine manufacturers to reduce or eliminate thimerosal in vaccines [Centers for Disease Control and Prevention, 1999; Institute of Medicine Immunization Safety Review Committee, 2001]. By 2002, all routinely recommended early childhood vaccines were thimerosal free.

The vast majority of studies have indicated no association between thimerosal containing vaccines and autism [Peltola et al., 1998; DeWilde et al., 2001; Kaye et al., 2001; Stratton et al., 2001; Madsen et al., 2002; Hviid et al., 2003; Jick and Kaye, 2003, 2004; Nelson and Bauman, 2003; Institute of Medicine, 2004; Parker et al., 2004; Honda et al., 2005], however, concerned families, often fueled by class action litigators and scare stories in the popular press [Kennedy, 2005], remain convinced that ethylmercury causes autism and insist collusion exists between the federal government health agencies, namely the FDA and CDC, the American Academy of Pediatrics and the drug companies which produce pediatric vaccines [Bernard et al., 2001, 2002; Holmes et al., 2003; Rimland, 2004; Geier and Geier, 2005; Safe Minds Homepage, <http://www.safeminds.org>]. A recent survey of families of children with autism found that though 90% of parents believed that genetic influences contributed to autism, 40% also thought vaccines contributed to or were responsible for their child's autism [Mercer et al., 2006]. As Mercer et al. point out, "the need to understand and make sense of a disability or serious illness is a critical coping mechanism, and thus, it is not surprising that for autism, . . . parents attempt to find explanations to facilitate dealing with the diagnosis".

To address the question of whether thimerosal exposure causes autism, we investigated thimerosal exposure during pregnancies which resulted in the birth of a child subsequently diagnosed with autism. Rh negative (Rh⁻) women are routinely treated with Rh immune globulin (RhIg) at 28 weeks gestation and earlier if bleeding or other symptoms indicate mixing of potentially Rh⁺ fetal blood in the mother's circulation. Since most RhIg manufactured in this country contained thimerosal until 2001, and since young fetal brains are more susceptible to neurotoxic effects, we hypothesized that if thimerosal were associated with the development of autism, we would find a higher proportion of Rh⁻ mothers of children with autism, born before 2002. To test this hypothesis, we assessed Rh status and ante partum thimerosal exposure of mothers and children who came to a statewide, dedicated autism clinic.

An additional consideration was whether we could identify a subgroup of children with autism who might be more sensitive to ethylmercury exposure. Over the last decade, our center has focused on identifying features which split autism into biologically discrete subgroups, such as physical dysmorphism, head size, type of onset and family history [Miles and Hillman, 2000; Miles et al., 2000, 2004, 2005; Stoelb et al., 2004]. By analyzing Rh status and antepartum thimerosal exposure, we addressed whether any of these subgroups could be sensitive to ethylmercury.

MATERIALS AND METHODS

The Autism Clinic at University of Missouri—Columbia, supported partially by the Missouri Department of Mental Health, was started in 1995 with dual missions of providing comprehensive wrap around care for individuals with autism and discovering more about causes and outcomes. To ensure an unbiased study population for our research mission, the program was designed to obviate biases of ascertainment. Inclusion criteria specified that all children and adults referred with questions of diagnosis, treatment including medical, educational and behavioral issues, and/or recurrence risk assessment be entered into the study population. No individuals meeting those criteria were excluded. Families come primarily from Missouri and all were enrolled in an IRB compliant study which enables us to analyze data we collect and to recontact families for subsequent studies. Since the clinic has no biases for or against specific treatment modalities or approaches, because no families who come through the clinic are excluded from study, and since no families are included who are referred because of research interests, we believe that biases are limited to families' desire for an evaluation. Support of the Missouri Department of Mental Health ensures that no families are turned away, reducing financial biases as well.

Clinical Evaluation

The Autism Clinic evaluation utilizes a standard data set for the collection of prenatal and perinatal history, development, language, behavior, health history, medication, dietary, metabolic, neurologic, and family history. Physical examinations are performed by trained dysmorphologists and include standard morphologic measurements of the head, face, hands, feet, body proportions, and dermatoglyphic analysis [Hall et al., 1989; Aase, 1990; Jones, 1997]. Skin is examined with a Woods lamp to assess for tuberous sclerosis. The occipital-frontal circumference is measured and microcephaly is defined as a measurement ≤ 2 nd centile and macrocephaly as ≥ 98 th centile. Based on the physical examination,

each patient is classified as dysmorphic or nondysmorphic as previously described [Miles et al., 2000, 2005]. Individuals who are dysmorphic or have microcephaly are designated as having complex autism, based on the premise that morphologic evidence of an insult to embryological development places them in a different etiological category than children with no such evidence. Approximately 20% of children evaluated in our clinic have complex autism, the remainder is considered to have essential autism [Miles et al., 2005]. Laboratory testing included high resolution karyotype, DNA for fragile X, urine metabolic screen, organic acids, urine amino acids, short chain fatty acids, TSH, FT₄, comprehensive metabolic profile, heme profile/differential, lead level, and EEG. Brain MRIs were offered and done in 67%. Because Rh blood group status is correlated with ethnic background, each mother's ethnic background was obtained. Mother's ethnic background was self reported on the initial Autism Family History form and verified by the geneticist who obtained the family history.

Autism Diagnostic Criteria

DSM-IV criteria and our center-based version of the ADI scoring protocol were used for the diagnosis of an ASD for all subjects. Independent diagnostic evaluations were conducted by a child psychiatrist and/or a neuropsychologist in most cases; if there was disagreement the results were discussed jointly to reach a consensus diagnosis. CARS scores were available on most patients either from school testing or as part of the neuropsychological evaluation. A subset of 93 patients were evaluated by the ADI-R [Lord et al., 1994], and in all cases the ADI-R confirmed the previous ASD diagnosis. Of the 214 children in the primary study group, 69.6% were diagnosed with autistic disorder, 8.4% with Asperger syndrome, and 21.9% with pervasive developmental disorder not otherwise specified (PDD-NOS) (Table III).

IQ Assessment

Each patient was assigned an IQ/DQ score based on the most recent and comprehensive neuropsychological evaluation. Children were evaluated by the Autism Center's neuropsychology team or recent results from the schools or other psychologists were used. When more than one set of test results were available, non-verbal IQ scores were used with the order of preferred testing being the Leiter-R [Roid, 1997], the WISC-III [Wechsler, 1991], and the Stanford Binet [Thorndike et al., 1986]. For younger children, developmental quotients were used based on standard scores from the daily living domain of the Vineland [Sparrow et al., 1984].

TABLE I. Demographic Characteristics

	Study group/records reviewed N = 214		Lost to follow-up N = 220		P value
	%	#	%	#	
Ethnicity ^a					
Caucasian	95.8	205/214	92.3	203/220	0.05
African-American	2.8	6/214	3.2	7/220	
Asian	0	0/214	3.2	7/220	
Hispanic	1.4	3/214	1.4	3/220	
Socio-economic status ^b					
Group I and II	44.8	86/192	48.1	79/164	0.73
Group III	29.2	56/192	25.6	42/164	
Group IV and V	26.0	50/192	26.2	43/164	
Mean age (SD)	7.2 (4.3)		8.2 (6.1)		0.2
Range	1.4–23.5		1.0–39.8		

^aEthnic background is of the mother.

^bSES was unknown for 10.3% of the study group and 25.4% of the lost to follow-up group.

Study Population

Between 1995 and 2005, 765 consecutive individuals were evaluated; 626 (81.8%) met diagnostic criteria for an autistic disorder, Asperger syndrome or PDD. From the 626 individuals who met the diagnostic criteria, we identified 525 families with at least one available parent. Children in adoptive, group or foster homes, deceased children, and adult individuals whose mothers were unavailable were excluded.

Three hundred five mothers who were reached by telephone agreed to answer a short list of questions about their blood group status and pregnancy exposures to RhIg. Only 19 mothers declined to participate, 180 families could not be located. Participating families were sent release of

information forms allowing us to send for pregnancy, newborn and childhood immunization records; we successfully received all records for 214 mothers of 230 ASD pregnancies. In multiplex families, the first child evaluated was designated the index case or if assessed together, the oldest child.

Demographics

Table I presents the age, socio-economic status, and ethnicity of the participating and non-participating families. Ethnicity was based on the mothers' background. Socio-economic status was by the Hollingshead method [Hollingshead, 1975] with Group I being the highest socio-economic level. The only statistical difference between the participating families and those lost to follow-up was the

TABLE II. Blood Group, Ante Partum RhIg, and Rh Incompatibility Data

	Rh ⁻	RhIg/thimerosal	Rh incompatibility (Rh+ fetus/ Rh ⁻ mother)
Study Group ^a	15.4%, (33/214)	13.9% ^b , (29/208)	60.6%, (20/33)
Comparison Group I ^c	15.4%, (10/65)	14.8% ^b , (4/27)	50%, (5/10)
P-value	0.99	0.22	0.55
Odds ratio	1.00	0.93	1.54
Confidence interval	0.47–2.16	0.30–2.89	0.37–6.38
Comparison Group II ^d	15.2%, (1,896/12,454)	—	61% expected ^e
P-value	0.94	—	0.96
Odds ratio	1.02	—	1.04
Confidence interval	0.7–1.48	—	0.48–2.24
Comparison Group III ^f	17.7%, (28,312/160,276)	—	57.9% expected ^e
Caucasian	18.5%	—	—
African-American	7.5%	—	—
Hispanic	9.0%	—	—
Asian	2.6%	—	—
P-value	0.40	—	0.75
Odds ratio	0.85	—	1.039
Confidence interval	0.61–1.27	—	0.48–2.24

^aData on 214 mothers of index case pregnancies; 16 with 2 ASD children.

^bRestricted to pregnancies prior to 2002.

^cSixty-five mothers of children with a de novo chromosome disorder.

^dUniversity of Missouri Hospital Rh typed patients 2005–2006.

^eExpected, based on Hardy–Weinberg calculation.

^fAmerican Red Cross—Missouri-Illinois Region.

families of Asian origin, which is not considered pathophysiologically significant.

Comparison Populations

A number of populations where Rh status was identified were used for comparison (Table II). The principal control group was recruited from families of children with Down syndrome and other de novo chromosome disorders who sought care from Medical Genetics at the University of Missouri; complete records were available from 65 families. This control group is appropriate because it controls for unappreciated biases related to how parents decide to bring their child to a University of Missouri Clinic and because autism was excluded. Second, we queried the University Hospital Blood Bank to determine the Rh status of patients blood typed at the University Hospital from the April 1, 2005 through March 31, 2006. This is considered our best estimate of Rh status in our region; ethnic background data, however, are not available. The third population was accessed from the Missouri-Illinois Red Cross and represents all individuals who donated blood in calendar year 2005; ethnicity is self-reported. Each of these comparison groups provide data from an equivalent Missouri catchment area. In addition, United States reference numbers were obtained from the literature and the American Red Cross [Mollison et al., 1993; Petrides and Stack, 2001; Brecher, 2005; American Red Cross Homepage, <http://www.givelife.org>]. Based on the Missouri-Illinois Red Cross data, we calculated expected Rh⁻ rates for our population's ethnic mix to be 18%. Based on United States American Red Cross numbers, the Rh⁻ percentage expected is 17.1%.

Statistical Analysis

To assess the association between ASD and mother's Rh status and exposure to ante partum thimerosal either the Chi-square test or Fisher's Exact test for small sample size were used and odds ratios were calculated. The Wilcoxon Rank Sum test was used to determine any differences between the age distribution of the study group and the group lost to follow-up. Hardy-Weinberg calculations were used to estimate the expected number of incompatible pregnancies for the 33 Rh⁻ mothers, assuming the fathers' Rh status was either similar to the mothers or similar to the University Hospital and Missouri-Illinois Region Red Cross data. Rh incompatibility was compared to expected, using the one sample test of proportions.

RESULTS

The study group consisted of 214 mothers of children diagnosed with an autistic disorder, Asperger syndrome or PDD-NOS between 1995 and 2005.

The Rh status, RhIg with thimerosal exposure, and Rh incompatibility were established by review of medical records (Table II). There were 33 Rh⁻ mothers (15.4%) which is not different from the 15.4% Rh⁻ mothers of children with sporadic chromosome disorders ($P=0.99$; odds ratio = 1.000, 95% CI: 0.47–2.16). It is also not different from the 15.2% Rh⁻ patients tested in University of Missouri Hospital patients ($P=0.94$; odds ratio = 1.02, 95% CI: 0.7–1.48) or the 17.7% Rh⁻ Missouri Illinois Regional Red Cross blood donors ($P=0.40$; odds ratio = 0.85, 95% CI: 0.61–1.27).

Of the 33 Rh⁻ mothers, 29 (88%) were treated with RhIg during their pregnancy. Four Rh⁻ mothers did not receive RhIg; one had no prenatal care, two were not offered RhIg, and in one the father was known Rh⁻. RhIg given to all 29 mothers contained thimerosal, which is 13.9% of 208 mothers who were pregnant prior to 2002. This is not statistically different from the 14.8% in the control group pregnant prior to 2002 ($P=0.22$; odds ratio = 0.93, 95% CI: 0.30–2.89) or the 15.4% (10/65) treated with RhIg with or without thimerosal ($P=0.94$; odds ratio = 1.02, 95% CI: 0.70–1.48).

None of the pregnancies received more than one ante partum dose of RhIg either by mother's report or review of the records. One mother received RhoGam following amniocentesis at 16 weeks and delivered prior to 28 weeks. When there was any question about the brand of RhIg used the hospitals or obstetrical offices were called to clarify the information. In all cases the product used was RhoGam. None of the Rh⁻ mothers were Rh sensitized.

Of the 33 Rh⁻ pregnancies, 20 (60.6%) were Rh incompatible. Hardy-Weinberg calculations were used to estimate the expected number of incompatible pregnancies for the 33 Rh⁻ mothers. Assuming the fathers' Rh status was similar to the mothers the expected proportion of Rh incompatible pregnancies was 61%. The percentage of Rh incompatible pregnancies in the control group was 50% ($P=0.55$; odds ratio = 1.54, 95% CI: 0.37–6.38). Similar calculations based on University of Missouri patient data and Missouri-Illinois Red Cross Rh status, indicated that 61% and 57.9% of pregnancies respectively would be expected to be Rh incompatible. For all comparisons, the proportion of Rh incompatible pregnancies in the study group was not statistically different from expected (Table II).

Sixteen of the 214 mothers had two children with ASD; 15 of those mothers were Rh⁺ and 1 was Rh⁻. When data were calculated based on all 230 ASD children, 14.8% (34/230) of mothers were Rh⁻, and 13% (30/230) received RhIg with thimerosal, which is almost identical to data calculated for index cases and does not differ statistically from any of the comparison or reference populations.

Our primary statistical analyses were based on comparisons to children with de novo chromosome

disorders. University Hospital Blood Bank and the Missouri-Illinois Red Cross data for Rh status by ethnic group were also compared. Comparison of the Rh status to the comparison populations revealed no statistically significant differences; the minimum significance level for the Chi-square tests of association was 0.46 and all 95% confidence intervals for odds ratios include the null value of 1.0.

Ninety-one mothers completed only the telephone survey and either did not return the release of information forms or records could not be obtained from their health care providers. This group did not differ from the primary study group or unreachable group in any of the demographic characteristics (data not shown). Of the mothers who only completed the telephone survey, a slightly higher percentage reported their blood type as Rh⁻ (19.8% vs. 15.8%). Though comparisons between the telephone report only group and the control populations did not generate statistically significant differences, we consider this data biased by the exclusion of 25 mothers (27.5%) who did not know their blood type. Women who have undergone a pregnancy are more likely to remember their blood type if they tested Rh⁻ because of the ensuing discussions of Rh incompatibility that only occur with the Rh⁻ mothers. This will bias recall studies toward a greater percentage of Rh⁻ mothers.

The second aim of the study was to determine whether Rh blood group status or exposure to antepartum thimerosal correlated with any specific subset of autism patients. Table III depicts the blood group frequencies, antepartum RhIg/thimerosal exposure and Rh incompatibility for ASD children separated on the basis of eight characteristics we have previously found to effect outcome and/or recurrence risks: (1) the ASD clinical diagnoses (autistic disorder, Asperger syndrome, and PDD-NOS), (2) IQ, (3) gender, (4) essential versus complex phenotype, (5) dysmorphology status, (6) head size, (7) regressive versus early onset ASD, and (8) multiplex versus singleton. Birth order was analyzed because of the higher risk of Rh sensitization in second and later pregnancies. These groups are not mutually exclusive. The Rh blood groups status, ante partum RhIg/thimerosal exposure and Rh incompatibility did not differ statistically from expected for any of the subgroups.

DISCUSSION

We hypothesized that if the increased prevalence of ASDs were due to exposure of children to ethylmercury in doses received with childhood immunizations, exposure at 28 weeks gestation would pose an even greater risk based on increased neural vulnerability early in development; and consequently mothers of children with autism would be more likely to be Rh⁻ and to have received

RhIg with thimerosal during pregnancy. Our survey of 214 families of children with an ASD revealed that Rh⁻ status was no higher in mothers of children with autism than in the general population, exposure to antepartum RhIg, preserved with thimerosal is no higher and pregnancies were no more likely to be Rh⁻ incompatible. This was also true for autism subgroups defined by behavioral phenotype, gender, IQ, regressive onset, head circumference, dysmorphology, birth status, essential, or complex phenotype. Thus, our findings support the consensus that exposure to ethylmercury in thimerosal is not the cause of the increased prevalence of autism.

In 1968, Anti-D RhIg was introduced for prophylaxis against hemolytic disease of the newborn, due to rhesus (D) alloimmunization; it is now the standard of care for Rh⁻ mothers given first at 28 weeks gestation and again after delivery of an Rh⁺ infant [ACOG, 1999; Royal College of Physicians, 1997]. With the use of RhIg, the maternal RhD sensitization rate in Rh⁻ pregnancies fell from 13.2% to 0.14% [Bowman, 2003] and the number of neonatal deaths from RhD HDN dropped from 0.018% in 1977 to 0.0023% by 1992 [Clarke and Hussey, 1994]. When widely applied, ante partum immunoprophylaxis can be expected to reduce the frequency of anti-D in pregnancy to 0.01% or less [Mollison et al., 1993] and almost completely obviate severe hemolytic disease requiring newborn exchange transfusion [Howard et al., 1998]. RhIg was first licensed by Ortho Clinical Diagnostics under the brand name RhoGam and has continued as the predominant product in the United States accounting for 70–90% of the market. Similar to vaccines and other immune globulin preparations, RhoGam was preserved with thimerosal (0.003% concentration) until 2001. Two other RhIg products, BayRho, produced by Bayer, and WinRho, produced by Cangene, supply the rest of the United States. BayRho contained thimerosal at a higher concentration (0.01%) from 1971 to 1996 and subsequently was produced without preservative. WinRho is produced by a freeze-dried method and never contained thimerosal [USDA Food and Drug Administration, Department of Health and Human Services, 2004]. Because of the variety of products and dosages, it is necessary to know exactly which product was used to determine thimerosal exposure.

In addition to this study, numerous groups have addressed safety of infant immunizations and whether there is any causal association with autism. The vast majority of research has not uncovered any evidence for a vaccine cause for autism [Peltola et al., 1998; DeWilde et al., 2001; Kaye et al., 2001; Madsen et al., 2002; Hviid et al., 2003; Jick and Kaye, 2003, 2004; Nelson and Bauman, 2003; Parker et al., 2004; Honda et al., 2005; Rutter, 2005; Fombonne et al., 2006]. Specifically, this is true for organic ethylmercury, where epidemiologic studies have failed to identify any concordance between thimerosal

TABLE III. Blood Group, Ante Partum RhIg With Thimerosal, and Rh Incompatibility in Autism Subgroups

Subgroups ^a	Rh ⁻			RhIg/thimerosal			Rh incompatibility			
	% (#)	P-value		% (#)	P-value		% (#)	P-value		
		Group I ^b	Group II ^c		Group III ^d	Group I ^b		Group II ^c	Group I ^b	Group II ^c
ASD Diagnosis										
Autistic disorder	14.1 (21/149)	0.81	0.70	0.25	12.8 (19/149)	0.22	71.4 (15/21)	0.24		
Asperger syndrome	33.3 (6/18)	0.09	0.03	0.08	33.3 (6/18)	0.10	50 (3/6)	0.39		
PDD-NOS	12.8 (6/47)	0.70	0.64	0.37	8.5 (4/47)	0.21	33.3 (2/6)	0.33		
IQ ^e										
IQ ≥ 70	18.7 (14/75)	0.61	0.41	0.82	18.7 (14/75)	0.22	64.3 (9/14)	0.48		
IQ 55-69	9.1 (3/33)	0.17	0.14	0.09	3 (1/33)	0.11	33.3 (1/3)	0.44		
IQ < 55	11.4 (4/35)	0.21	0.17	0.12	8.8 (3/34)	0.24	75 (3/4)	0.34		
Gender										
Male probands	15.9 (27/169)	0.91	0.79	0.57	14.8 (25/169)	0.23	55.6 (15/27)	0.76		
Female probands	13.3 (6/45)	0.76	0.72	0.45	8.9 (5/45)	0.26	83.3 (5/6)	0.19		
Essential vs. Complex ^f										
Essential ASD	14.2 (22/155)	0.82	0.72	0.26	12.9 (20/155)	0.22	54.5 (12/22)	0.81		
Complex ASD	21.4 (6/28)	0.48	0.36	0.60	17.9 (5/28)	0.27	83.3 (5/6)	0.19		
Dysmorphology ^g										
Nondysmorphic	14.6 (23/157)	0.21	0.84	0.32	13.4 (21/157)	0.23	56.5 (13/23)	0.73		
Dysmorphic ^h	19.2 (5/26)	0.66	0.17	0.82	15.4 (4/26)	0.30	80 (4/5)	0.25		
Equivocal	16 (4/25)	0.25	0.21	0.21	12 (3/25)	0.3	75 (3/4)	0.34		
Head size ⁱ										
Macrocephaly	17.2 (10/58)	0.78	0.81	0.93	17.2 (10/58)	0.24	40 (4/10)	0.32		
Normocephaly	13.8 (20/145)	0.76	0.63	0.22	11.7 (17/145)	0.21	70 (14/20)	0.28		
Microcephaly	40 (2/5)	0.17	0.14	0.17	40 (2/5)	0.19	100 (2/2)	0.32		
Onset type										
Regressive onset	12.5 (9/72)	0.63	0.52	0.25	88.9 (8/72)	0.23	66.7 (6/9)	0.28		
No regression	16.9 (24/142)	0.78	0.58	0.81	87.5 (21/142)	0.23	58.3 (14/24)	0.66		
Multiplex vs. simplex ASD										
Simplex	16.2 (32/198)	0.88	0.72	0.58	14.6 (29/198)	0.23	62.5 (20/32)	0.48		
Multiplex	6.3 (1/16)	0.24	0.21	0.15	0 (0/16)	0.14	0 (0/1)	0.55		
First vs. later born sibs										
First born	16.1 (20/124)	0.89	0.78	0.65	10.5 (13/124)	0.2	60 (12/20)	0.60		
Later born	15.6 (14/90)	0.98	0.93	0.60	15.6 (14/90)	0.24	57.1 (8/14)	0.73		

^aDenominator is number of families in each subgroup; index case used for multiplex families.

^bCompared with Comparison Group I.

^cCompared with Comparison Group II.

^dCompared with Comparison Group III.

^eProbands with unknown IQ = 71.

^f1 probands were equivocal for essential/complex designation.

^g5 unclassified for dysmorphology; 2 syndromes in nondysmorphic group, 5 in dysmorphic group and 1 in mild group.

^h3 in dysmorphic group had microcephaly.

ⁱHead size could not be measured for 6 probands, but appeared normocephalic.

exposures and autism [Madsen et al., 2002; Fombonne et al., 2006]. One disquieting report came from animal studies, which showed that a known mercury sensitive strain of mice, experienced neurological deficits after injection with small doses of mercury, comparable to those received in infant immunizations [Hornig et al., 2004]. This raised the question of whether a small group of genetically sensitive infants could be harmed.

Despite evidence that autism is primarily a genetic disorder [Gillberg, 1998; Keller and Persico, 2003; Muhle et al., 2004], concern about environmental or teratogenic etiologies is warranted. A number of fetal teratogens have been identified, beginning with fetal rubella in the 1960s, fetal exposure to valproic acid, usually prescribed to control maternal seizures, thalidomide and possibly others [Arndt et al., 2005]. Though these cases represent a small proportion of all children with autism, scientists are beginning to consider whether epigenetic modulation in gene expression could be teratogen responsive [Fombonne, 2003; Jaenisch and Bird, 2003; Keller and Persico, 2003; Egger et al., 2004; Singh et al., 2004; Hong et al., 2005]. There is evidence that epigenetic mechanisms may affect behavior and social outcomes. For instance, maternal behavior toward young offspring affects the size of the offspring's hippocampus in adulthood [Weaver et al., 2004, 2005; Szyf et al., 2005] and social deprivation in infancy can lead to persistent suppression of the oxytocin and arginine vasopressin peptide systems [Fries et al., 2005]. DNA methylation of the promoter regions of genes appears to be an initiating event for an epigenetic cascade. A number of environmental factors which influence DNA methylation have been identified including antioxidants, vitamins involved in methylation pathways (folate, B6 and B12), zinc, iron, magnesium, manganese, selenium, arsenic, viral infections, prenatal, and early postnatal nutrition [Waterland and Jirtle, 2004; Rodenhiser and Mann, 2006]. Mercury, however, is not on the list of agents currently known to affect DNA methylation.

Few studies have focused on pregnancies of Rh⁻ mothers who received RhIg at 28 weeks gestation, presumably because the thimerosal dose injected intramuscularly to the mother is significantly diluted before reaching the fetus and therefore has been assumed by researchers and funding agencies to be innocuous. Pichichero et al. [2002] reported that the concentration of thimerosal in infants' blood following administration of thimerosal containing vaccines was in the nanomolar range; hence, the fetal dose following maternal injection would be well below levels considered toxic. Nevertheless, there is fear that even very small doses, delivered when the brain is more sensitive to mercury exposure could be toxic. Numerous internet sites and one research study assert that antenatal Rh immune prophylaxis causes autism and that a high percentage of mothers of

children with autism are Rh⁻. Holmes et al. [2003] reported that 46% of mothers of 94 children with autism were Rh⁻ and received ante partum RhIg. Their data were obtained from families who came from all parts of the United States to a clinic that advocated chelation therapy, without doubt creating an ascertainment bias toward Rh⁻ mothers who received RhIg. In contrast, we found that of families evaluated in a large Missouri autism clinic which espoused no specific treatment paradigms, only 15.4% of mothers were Rh⁻. Moreover, Rh status in the Holmes study was self-reported, which we have shown also biases results in favor of Rh⁻ recall.

Of the 33 Rh⁻ mothers in this study, 29 (88%) received ante partum RhIg with thimerosal, 3 Rh⁻ mothers were untreated due to inadequate prenatal care, and for 1 the father was Rh⁻. Our finding that 88% of Rh⁻ mothers received RhIg is considered average, and indicates that though only Rh⁻ women receive RhIg, not all Rh⁻ ASD mothers are in fact exposed to thimerosal. Thus, our finding that only 13.9% of mothers of children with ASD received ante partum thimerosal is realistic and no higher than expected for mothers of similar age children.

A recent study questioned whether Rh maternal-fetal incompatibility itself, distinct from the issue of RhIg treatment with or without thimerosal could increase the risk for autism and found no association [Zandi et al., 2006]. Earlier studies had suggested this could be a risk factor for schizophrenia spectrum disorders [Palmer et al., 2002; Kraft et al., 2004]. Studying a cohort of births occurring between 1959 and 1967, prior to anti-Rh prophylaxis, Insel et al. [2005] showed the adjusted incidence rate ratio of schizophrenia was 1.80 for Rh incompatible compared with Rh compatible pregnancies and males had a higher rate ratio (RR = 2.37). They postulated that maternal-fetal incompatibility causes a maternal immune response to the paternally inherited Rh+ antigen, which subsequently disrupts fetal neurodevelopment. We, however, found no increase over expected in the proportion of Rh incompatible pregnancies for children with ASD and no difference for any of the ASD subgroups including comparisons by gender and birth order.

Because the ASDs are clinically and etiologically heterogeneous [Miles and McCathren, 2005], it has been suggested that a vulnerable subgroup of children with autism might be affected by low dose exposures during pregnancy. In response to concern that only certain groups of children are at risk, we analyzed specific ASD subgroups; none had statistically significant increases in either Rh negativity or thimerosal exposure during pregnancy. Mothers of children with microcephaly and Asperger syndrome had the highest Rh⁻ and thimerosal exposure; of five microcephalic children, two mothers (40%) were Rh⁻ and received ante partum RhIg. Of the 18 subjects with Asperger, 6 mothers (33.3%) were Rh⁻ and

received RhIg. We analyzed them to determine whether there was any pattern that could relate thimerosal exposure to cause; the examination revealed no consistencies. Microcephaly is the most specific indicator of a poor outcome in autism [Miles et al., 2000]. Review of these three subjects indicated that each met criteria for poor outcome. Asperger syndrome on the other hand is diagnosed on the basis of normal or near normal language and is a good prognostic sign for independent functioning [Ulm et al., 1999]. Review of records from the six Asperger subjects indicated all were high functioning. Thus, we feel comfortable that these two subgroups with the highest proportions of Rh⁻ mothers and RhIg treatment merely reflect small sample sizes resulting from subdividing the study population.

The primary limitation of this study is the relatively small number of subjects, especially those who were Rh⁻ and received ante partum RhIg which contained thimerosal. It is however the largest study reported and demonstrates how ascertainment biases and recall reports can mislead. An additional constraint is the small age matched non-ASD control population. To access larger populations, we utilized University Hospital records of Rh status and regional Red Cross data; unfortunately the utility of those populations is lessened by the inability to identify individuals with an ASD disorder. If the frequency of ASDs is as high as 1 in 175, we would expect 71 of the 12,454 individuals' blood typed at University Hospital to also have an ASD; removing all 71 from either the Rh⁻ or Rh⁺ groups did not affect the results.

All medical advances raise questions of risk and safety which must be addressed; this is especially important for the ASDs where precise etiologies have not been determined and anxiety about possible epidemics is palpable. This study adds to the evidence that there is no causal association between thimerosal and childhood autism. Though RhIg and routine childhood vaccines are now thimerosal free in the United States, continuing to analyze questions of safety remains important. We hope this report of no association between autism, Rh negativity, and thimerosal exposure during pregnancy will offset some of the decreased compliance with immunization recommendations which is known to increase morbidity and mortality from childhood infectious diseases [Klin, 2003]. Moreover, thimerosal continues to be used in many places around the world to preserve multi-dose vials which in turn makes vaccines affordable; it is important that health authorities have valid scientific data on which to base public health decisions. Finally, it is important for families of children with ASDs and especially those who received ante partum RhIg with thimerosal to have this information.

ACKNOWLEDGMENTS

We thank Rebekah Algya Snell for data acquisition and management and Gregory Petroski, Ph.D., Biostatistician, Division of Medical Research, University of Missouri School of Medicine for statistical advice and oversight. This study was supported by a grant from Johnson and Johnson Company and ongoing autism research support from the Leda J. Sears Trust. Neither funding source was involved in the design and conduct of the study, collection, management, analysis or interpretation of the data, preparation, review, or approval of the manuscript.

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