

## Thimerosal Exposure in Infants and Developmental Disorders: A Retrospective Cohort Study in the United Kingdom Does Not Support a Causal Association

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**ABSTRACT.** *Objective.* After concerns about the possible toxicity of thimerosal-containing vaccines in the United States, this study was designed to investigate whether there is a relationship between the amount of thimerosal that an infant receives via diphtheria-tetanus-whole-cell pertussis (DTP) or diphtheria-tetanus (DT) vaccination at a young age and subsequent neurodevelopmental disorders.

*Methods.* A retrospective cohort study was performed using 109 863 children who were born from 1988 to 1997 and were registered in general practices in the United Kingdom that contributed to a research database. The disorders investigated were general developmental disorders, language or speech delay, tics, attention-deficit disorder, autism, unspecified developmental delays, behavior problems, encopresis, and enuresis. Exposure was defined according to the number of DTP/DT doses received by 3 and 4 months of age and also the cumulative age-specific DTP/DT exposure by 6 months. Each DTP/DT dose of vaccine contains 50 µg of thimerosal (25 µg of ethyl mercury). Hazard ratios (HRs) for the disorders were calculated per dose of DTP/DT vaccine or per unit of cumulative DTP/DT exposure.

*Results.* Only in 1 analysis for tics was there some evidence of a higher risk with increasing doses (Cox's HR: 1.50 per dose at 4 months; 95% confidence interval [CI]: 1.02–2.20). Statistically significant negative associations with increasing doses at 4 months were found for general developmental disorders (HR: 0.87; 95% CI: 0.81–0.93), unspecified developmental delay (HR: 0.80; 95% CI: 0.69–0.92), and attention-deficit disorder (HR: 0.79; 95% CI: 0.64–0.98). For the other disorders, there was no evidence of an association with thimerosal exposure.

*Conclusions.* With the possible exception of tics, there was no evidence that thimerosal exposure via DTP/DT vaccines causes neurodevelopmental disorders. *Pediatrics* 2004;114:584–591; cohort study, neurodevelopment, safety, thimerosal, thiomersal, vaccines.

**ABBREVIATIONS.** Hg, mercury; WHO, World Health Organization; VSD, Vaccine Safety Datalink; CDC, Centers for Disease

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Control and Prevention; HMO, health maintenance organization; ADD, attention-deficit disorder; GPRD, General Practice Research Database; ICD, *International Classification of Diseases*; DTP, diphtheria-tetanus-whole-cell pertussis; DT, diphtheria, tetanus; GP, general practitioner; HR, hazard ratio; CI, confidence interval.

Inorganic mercury (Hg) poses a potential risk of neurodevelopmental and renal toxicity in young children.<sup>1,2</sup> Cumulative exposure to an organic mercury-containing compound, methylmercury, can also produce neurologic or renal damage as it has a long half-life and can cross the blood-brain barrier, where it accumulates and is converted to inorganic mercury. Guidelines to limit cumulative exposure to methylmercury have been drawn up by various agencies and incorporate a wide margin of safety. The maximum daily dose specified by these different agencies varies by nearly 5-fold, the most stringent being the guideline of the Environmental Protection Agency in the United States that specifies a maximum daily exposure to Hg of 0.1 µg/kg extrapolated from data on methylmercury exposure. These guidelines are reproduced by Pichichero.<sup>2</sup>

Ethylmercury, a related organic mercury compound, is a constituent of thimerosal, an antibacterial agent used in certain nonlive vaccines. Ethylmercury has a much shorter half-life than methylmercury, being rapidly excreted via the stools after parenteral administration such that blood levels remain substantially below the safe threshold.<sup>2</sup> Nevertheless, the guidelines to limit cumulative methylmercury exposure have been translated to ethylmercury.<sup>3</sup> In the United States, increases during the 1990s in the number of childhood vaccines that contained thimerosal, which contains 49.6% Hg by weight, led to questions about safety because the maximum cumulative exposure in some US children was 187.5 µg Hg by 6 months of age, which would have exceeded the stringent Environmental Protection Agency limit. Although there is no evidence that this level of Hg exposure via ethylmercury was likely to or had actually caused any harm, a joint statement was issued by the American Academy of Pediatrics and the Public Health Service in 1999 recommending the removal of thimerosal from vaccines as soon as possible, as a precautionary measure.<sup>4</sup> Although the World Health Organization (WHO) supported in principle the move toward thimerosal-free vaccines, it nevertheless recommended that vaccines that contain thimerosal continue to be used in the meantime because the

Case 5:03-cv-00141-TJW Document 328-5 Filed 01/25/2005 Page 25 of 56  
 known morbidity and mortality from vaccine-preventable diseases greatly outweighed any theoretical risk from ethylmercury.<sup>5</sup>

In 2001, the preliminary results of an unpublished US cohort study that screened for associations between various neurodevelopmental and renal disorders and infant thimerosal exposure in vaccines were made available to an Institute of Medicine Immunization Safety Review.<sup>6</sup> This study used the computerized Vaccine Safety Datalink (VSD) developed by the Centers for Disease Control and Prevention (CDC) in association with 2 health maintenance organizations (HMOs).<sup>7</sup> The preliminary results suggested a possible trend between the level of ethylmercury exposure in the first few months of life and the following neurodevelopmental diagnoses: tics, attention-deficit disorder (ADD), language/speech delays, unspecified delays, and general neurodevelopmental delays. Although additional analyses were later conducted to control for confounding variables and to include more data, some disorders remained significant. Given the exploratory nature of this study, it was unclear whether these findings were real, a result of chance, or a result of uncontrolled confounding or bias. A subsequent, much smaller study by the CDC using another HMO data set did not confirm the first findings but had inadequate power to identify effects of the size seen in the first study.<sup>6</sup>

After review of the available evidence by the WHO Global Advisory Committee on Vaccine Safety, it was recommended that other studies be conducted to test the hypotheses raised by the VSD study.<sup>8</sup> The General Practice Research Database (GPRD) in the United Kingdom was identified as 1 of the few databases that were comparable to the HMO databases used in the VSD study.<sup>9,10</sup> In addition, the Avon Longitudinal Study of Pregnancy and Childhood in the United Kingdom was identified as a prospective cohort with information on vaccination and regular assessment of children's developmental progress. This cohort had the advantage of having data on many potential confounding variables, although it was not large enough to assess rare outcome conditions. The results of the analysis of this study are published together with this article.<sup>11</sup>

The GPRD holds data on all significant patient consultations, referrals, and prescribed medicines, including vaccines from 1988 from ~500 general practices in the United Kingdom. Together, these practices provide primary health care for 3.4 million patients (5.7% of the population). Preliminary analyses conducted by staff of the Morbidity and Health Care Team of the Office for National Statistics (which until 1999 managed the GPRD) using the *International Classification of Diseases* (ICD) codes for the outcomes of interest from the CDC study confirmed that the GPRD had sufficient power to test the hypotheses generated in the CDC study.

In the United Kingdom, the only vaccine that contains thimerosal and has been used routinely in the infant immunization program in the past 2 decades is diphtheria-tetanus-whole-cell pertussis (DTP) vaccine or diphtheria-tetanus (DT) vaccine and any com-

ination vaccine that contains DTP or DT. These vaccines all contain 50 µg of thimerosal (25 µg of Hg) per dose. No other thimerosal-containing vaccines have been given routinely to United Kingdom children, so the cumulative Hg exposure by age can be readily obtained from the number of doses of DTP- or DT-containing vaccines given. Because the United Kingdom changed to an accelerated 2/3/4 month DTP immunization schedule in 1990 (replacing the former 3/5/10 month schedule) and because vaccinations are generally given on time in the United Kingdom, a substantial proportion of children in the GPRD cohort will have had a cumulative Hg exposure of 150 µg of thimerosal (75 µg of Hg) by 4 months of age. This level of Hg exposure, although lower than the maximum of 187.5 µg received in the United States by 6 months of age, is similar to the level received by ~3 to 4 months of age in the United States. It is also the same as the amount of thimerosal used by developing countries that follow the expanded immunization schedule.

## METHODS

### The GPRD Cohort

Information on all children who were born from 1988 to 1997 and had at least 2 years of continuous follow-up from birth in the GPRD was obtained from the Office for National Statistics. Data were available up to the end of 1999 in linked patient, medical, and prevention databases for 152 898 children. For quantifying thimerosal exposure by age, it was important that an exact date of birth (to the day) be available. The patient database had information only on year and month of birth, but we were able to obtain exact dates of birth for 109 863 children from the date at which procedures or measurements taken on the day of birth were recorded in the linked medical database. Additional data quality processing, mostly concerning the validity of the dates of birth, vaccination, or the date of recording of the neurodevelopmental problems, led to the exclusion of 2711 records (2.5% of the cohort), leaving 107 152 children for analysis (Fig 1).

For each child, information was available on date of birth, gender, date leaving the practice (if applicable), last date that data were obtained from the practice, dates of all vaccinations (along with vaccine code and dose number), and dates and Read or OXSMIS codes for all medical events. Read and OXSMIS are diagnostic coding schemes that are built into practice software and based respectively on ICD-9 and ICD-8 codes. We had no information enabling identification of the patient and no information on general practitioner (GP) practice, so the only potential confounding variables that could be allowed for were gender and year/month of birth.

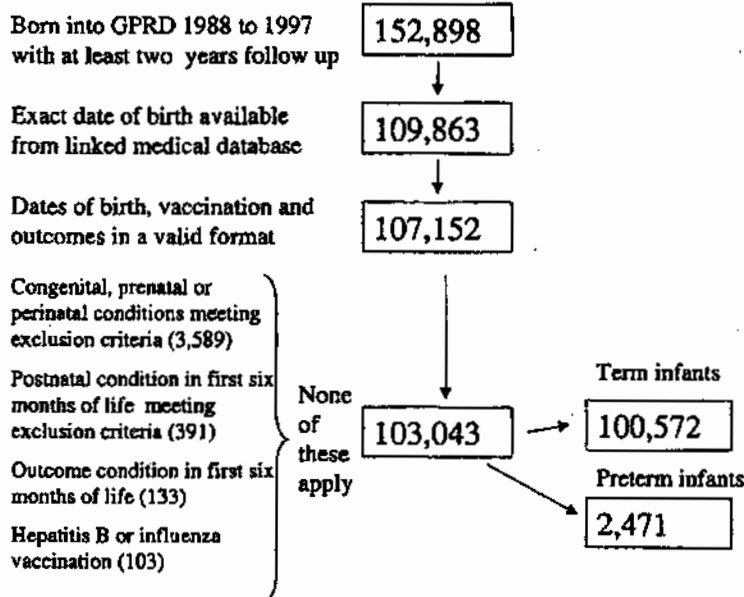
### Exclusion Criteria

Children with Read and OXSMIS codes relating to a variety of prenatal, perinatal, and postnatal conditions that occurred before 6 months of age were excluded as were children who were recorded as having an outcome event in the first 6 months of life. These children were excluded from the main analysis because the presence of such a condition is likely to affect both vaccination and future neurodevelopmental outcomes. Examples of exclusions were birth asphyxia, Down syndrome, cerebral palsy, meningitis, encephalitis, and head injury. Children were also excluded when they received either hepatitis B or influenza vaccination in the first 6 months of life because such children are likely to be an atypical subgroup. Children who were born preterm (<37 weeks' gestation) are likely to be of low birth weight, and many stay small. Such infants might be more susceptible to standard doses of thimerosal. Preterm infants therefore were analyzed separately.

### Exposure Variables

Hg exposure for each child was defined according to the number of DTP/DT doses received at 3 months (93 days) and 4 months

Fig 1. Selection of the GPRD cohort.



(124 days) of age. These ages were chosen to give a wide distribution for the number of children who received 0 to 3 doses of DTP/DT. A continuous variable (HgAll) that aimed to capture the age-specific Hg exposure up to 6 months (183 days) of age was also created. This variable was created to circumvent the problem of choosing age cut-offs and also to provide greater study power. HgAll was created from the age in days at the 3 DTP/DT doses as follows:

$$\text{HgAll} = [(183 - \text{age at dose 1}) + (183 - \text{age at dose 2}) + (183 - \text{age at dose 3})]/40$$

When a dose was not given or was given later than 183 days of age, for the purpose of the above calculation, the age was set to 183 days. The higher the value of HgAll, the earlier the 3 doses of DTP/DT were given and the child thus was exposed to a higher dose of mercury at a younger age. The arbitrary division by 40 was to ensure that when calculating hazard ratios (HRs), 1 unit of HgAll was of a meaningful size. One unit of HgAll corresponds to a combined difference of 40 days (while under the age of 183 days) in the age at which DTP/DT is given. For example, a child who received dose 1 at 60 days, dose 2 at 88 days, and dose 3 at 116 days would have an HgAll value of 7.125, whereas a child who received doses 1 and 2 by the same age but dose 3 at 156 days would have an HgAll value of 6.125.

**Outcome Events**

The outcome events of most interest were OXMS and Read codes relating to general neurodevelopmental disorders (a com-

posite category that comprised the following ICD-9 codes: 299 [childhood psychoses including autism], 300.3 [obsessive-compulsive disorders], 307 [specific psychopathological syndromes], 312.0 [unsocialized disturbance conduct, aggressive], 313 [emotional disturbance], 314 [hyperkinetic syndrome], 315 [specific delays in development], 317-319 [mental retardation], and V40 [mental and behavioral problems]) and other individual conditions as follows: unspecified development delays, tics, ADD and language or speech delay, enuresis, encopresis, autism, and non-specific behavioral problems. The ICD-9 codes relating to these outcomes are shown in Table 1.

**Statistical Methods**

The data were analyzed by Cox proportional hazards survival analysis in the statistical package S-Plus.<sup>12</sup> Survival for each child was taken as the number of days from age 183 days to the age at the first mention of each predefined outcome of interest. If for a particular outcome no event occurred, then survival was taken as being greater than the time to the end of follow-up. HRs with 95% confidence intervals (CIs) and two-sided P values were calculated for the effect of thimerosal exposure. The effect of the number of doses received by 3 and 4 months of age was quantified by the trend in hazard per dose. When the trend was significant, the HRs for 1, 2, and 3 doses at 4 months compared with the baseline of 0 doses were also calculated. A HR >1 is consistent with the hypothesis that early Hg exposure is associated with an increased risk of a predefined developmental outcome, whereas a HR <1 is

TABLE 1. Numbers With the Various Outcome Conditions for the Term and Preterm Cohorts, the Percentage Male, and the Estimated Median Age in Years at First Mention

Outcome (ICD-9 Codes)	Term Infants			Preterm Infants		
	n	% Male	Median age at First Mention, y	n	% Male	Median age at First Mention*, y
General developmental disorders	2035	71.1	3.6	110	66.4	3.6
Unspecified behavioral problem (3129)	816	71.2	4.8	30	70.0	5.3
Enuresis (7883)	1312	53.6	5.6	35	60.0	6.1
Encopresis (7876)	121	66.9	5.5	4	75.0	—
Tics (3072)	70	70.0	5.2	1	100.0	—
ADD (314)	222	77.0	3.7	8	87.5	—
Language/speech (3153)	666	70.4	3.0	33	69.7	3.4
Unspecified delay (3159)	485	67.2	2.4	52	59.6	2.1
Autism (2990)	104	89.4	4.4	2	50.0	—

\* Where there are <10 cases, a median age is not calculated

TABLE 2. Distribution in the Term and Preterm Cohorts of the Number of Doses of DTP/DT Received in Total, by 3 and 4 Months of Age

Exposure	Level	Term Cohort		Preterm Cohort	
		n	%	n	%
No. of doses of DTP/DT	0	945	0.9	37	1.5
	1	1687	1.7	38	1.5
	2	1090	1.1	60	2.4
	3 (third dose $\leq$ 1 y)	94730	94.2	2255	91.3
Doses by age 3 mo	3 (third dose >1 y)	2120	2.1	81	3.3
	0	7881	7.8	350	14.2
Doses by age 4 mo	1	51309	51.0	1390	56.3
	2	41382	41.1	731	29.6
	0	3419	3.4	142	5.8
Doses by age 4 mo	1	11766	11.7	442	17.9
	2	50349	50.1	1299	52.6
	3	35038	34.8	588	23.8

indicative of a potential protective effect. In all analyses, gender and year of birth were included as potential confounding factors; month of birth was also included when statistically significant at a 5% level. The effect of the number of doses of thimerosal was also examined visually in reverse Kaplan-Meier plots.

The main analysis included all children whether recorded as receiving 0, 1, 2, or 3 doses of DTP/DT at any age. However, it seemed possible that, as a result of socioeconomic or other confounding factors, children who did not complete vaccination in the first year of life would form a biased group. The data therefore also were analyzed after excluding all children who did not receive 3 doses of vaccination by age 366 days.

The median age at first mention of each outcome (Table 1) was estimated by taking the proportion of those who were followed up for 8 years or more with an event by 8 years (eg, 3.33% of 7195 followed up for at least 8 years had a general developmental disorder) and then finding the age by which half of this proportion had had an event (eg, 1.67% of 63 466 followed up for 3.6 years or more). This method of estimating the median age was used to adjust for the effect of censored data but is still conditional on the event occurring by the age of 8.

#### Validation

Validation of GP notes could be performed only for those GP practices that were still participating in the GPRD and with the case still registered. Validation was performed by sending a questionnaire to the GP asking for confirmation and additional details of the diagnosis and any subsequent related consultations and also the vaccination history, date of birth, and gender. Copies of relevant patient notes were also requested. Validation was sought for all cases of tics for whom validation was possible (36 of the 71 cases) as well as a random subset of 30 with ADD, 40 with language or speech delay, 30 with unspecified developmental delays, and also an additional 30 in the general developmental delay category not covered by the above.

## RESULTS

#### Cohort Selection

Details of the selection of the cohort of 103 043 children are given in Fig 1. The average length of follow-up in the cohort was 4.7 years (range: 2–11). Only 7.3% had a follow-up of longer than 8 years, reflecting that fewer practices contributed to the GPRD from 1988 to 1990.

#### Exposure

More than 96% of term children eventually received all 3 doses of DTP/DT (Table 2). By 4 months of age, most children had received 2 or 3 doses; however, there was sufficient variability in the number of doses received to enable fairly precise estimates of the trend in the HR per dose for the various

outcomes. Preterm children were less vaccinated and received vaccination later than term children.

Figure 2 shows the distribution of HgAll for the term cohort. The median value (interquartile range) of HgAll is 6.5 (4.5–7.0) in the term cohort and 6.1 (4.7–6.8) in the preterm cohort. Although few children received vaccinations early (HgAll >7.5), many got the 3 doses close to the correct time (HgAll: 6.5–7.5). Short delays in receiving the 3 doses were fairly common. However, relatively few children received <3 doses or got the vaccine very late.

#### Outcomes

All of the neurodevelopmental disorders investigated were more common in boys than in girls (Table 1). They also occurred more often in preterm children, with general developmental disorders occurring in 4.5% of preterm children and 2.0% of term children. The estimated median age of first mention of the disorders in term children varied from 2.4 years for unspecified delays to 5.6 years for enuresis. The age at first mention was similar for the term and preterm cohorts. Other than the general developmental disorders category, the most common disorders were enuresis, behavioral problems, and language/speech delays.

#### Risk Estimates

Table 3 shows the adjusted HRs per DTP/DT dose or HgAll unit for the various disorders. There were apparent protective effects from DTP/DT exposure for general developmental disorders, ADD, and unspecified developmental delay. The only evidence of a greater hazard with increasing thimerosal exposure was for tics, and this was significant only in the analysis that excluded children who did not receive 3 doses by 1 year of age. For the other disorders, exclusion of children who did not receive 3 doses by age 1 did not substantially affect the HRs; for example, the HR per dose at age 4 months was 0.86 (95% CI: 0.81–0.92) for general developmental disorders.

In the preterm cohort, none of the HRs was significantly different from 1 (data not shown). This cohort was not large enough to have the power to identify small effects; however, the direction of the effects was similar to the term cohort. For example, for

Fig 2. Distribution of the HgAll variable in the term cohort.

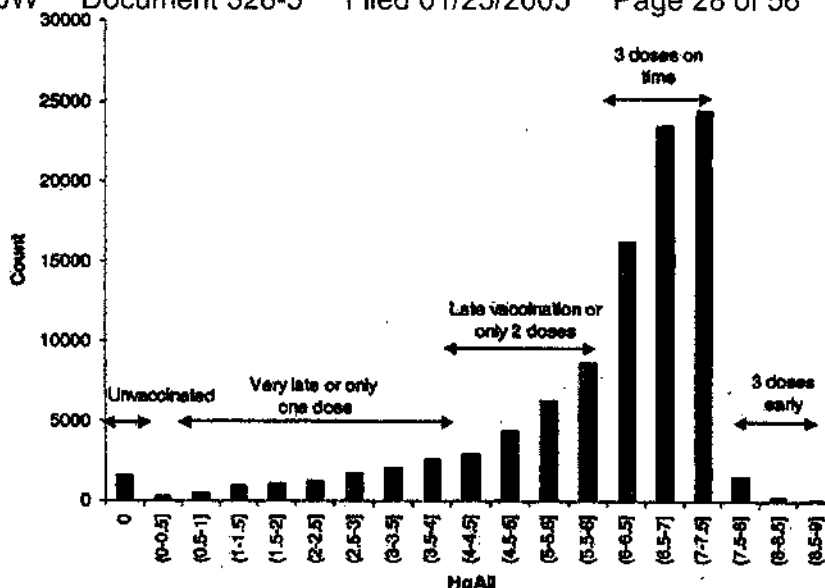


TABLE 3. HR for Various Neurodevelopmental Disorders According to the Number of Doses of DTP/DT Received by 3 and 4 Months of Age and the Age-Specific Cumulative Exposure HgAll in the Term Cohort

Outcome	Doses by 3 Months			Doses by 4 Months			HgAll		
	HR* Per Dose	95% CI	P Value	HR Per Dose	95% CI	P Value	HR Per Unit	95% CI	P Value
General developmental disorders	0.87	0.81-0.93	<.001	0.89	0.84-0.94	<.001	0.95	0.92-0.97	<.001
Behavioral problem	0.97	0.87-1.08	.55	0.98	0.90-1.07	.68	0.98	0.94-1.02	.36
Enuresis	1.07	0.98-1.17	.13	1.04	0.97-1.12	.25	1.02	0.98-1.05	.29
Encopresis	0.81	0.61-1.07	.13	0.82	0.65-1.02	.074	0.92	0.84-1.02	.11
Tics	1.45	0.99-2.15	.059	1.34	0.96-1.85	.082	1.14	0.97-1.35	.11
Ticst	1.62	1.05-2.50	.031	1.50	1.02-2.20	.035	1.33	1.06-1.69	.015
ADD	0.79	0.64-0.98	.033	0.82	0.70-0.97	.022	0.90	0.84-0.97	.004
Language or speech delay	0.89	0.79-1.01	.070	0.96	0.87-1.06	.38	0.99	0.94-1.03	.56
Unspecified developmental delay	0.80	0.69-0.92	.002	0.84	0.75-0.94	.002	0.91	0.86-0.95	<.001
Autism	0.89	0.65-1.21	.46	0.94	0.73-1.21	.66	0.99	0.88-1.12	.89

\* Adjusted for gender, year of birth, month of birth (general developmental disorders only).

† Results from the analysis that excluded those who did not receive 3 doses of DTP/DT by 366 days.

general developmental disorders, the HR per doses at 4 months was 0.80 (95% CI: 0.63-1.00). There was no evidence that the higher exposure by body mass in preterm children gave an increased risk of neurodevelopmental problems.

Table 4 shows the HRs of 1, 2, and 3 doses by 4 months of age compared with the baseline of 0 doses for variables with a significant trend by dose. The results show that for general developmental disorders, ADD, and unspecified delay, there is a decreasing trend by dose. For tics, the effect is less clear, with the main difference being the lower hazard at 1 dose. Reverse Kaplan-Meier plots show these results in more detail (Fig 3).

The 4109 children who were dropped as a result of the initial exclusion criteria were examined in a separate analysis. As with the premature children, they had a lower DTP/DT exposure than the main cohort and also a greater risk of outcome events. As with the term cohort, this group showed a protective DTP/DT effect for general developmental disorders

with a HR for the trend in doses by 4 months of age of 0.84 (95% CI: 0.72-0.97).

**Validation**

From the validation exercise, responses were received from 162 of 166 general practices. Of these, 10 could not provide any information. Of the remaining 152, 122 (80%) confirmed that the child presented with the given condition, 11 (7%) stated that the diagnosis reflected only parental concern, 11 (7%) had the diagnosis incorrectly coded, and in 8 (5%) no record of the diagnosis or subsequent episodes could be found in the notes. Of the 122 with a confirmed diagnosis, 48 were transient problems, 31 were long term, and for 43, the duration could not be determined. For tics, responses were received for all 36, of whom the duration of symptoms could be determined in 27. In 24 (89%) of 27, the tic was only a transient problem. In 3 cases, tics was recorded when in fact the individual presented with a parasitic tick. The validation confirmed that the dates of vaccina-

TABLE 4. Effect of Number of DTP/DT Doses Received by 4 Months of Age on Outcomes With Significant Associations in the Trend Analysis for the Term Cohort

Outcome	DTP/DT Doses by Age 4 Months	No. With Outcome	HR*	95% CI
General developmental disorders	0	86	1.00	Reference
	1	302	0.99	0.78-1.25
	2	1028	0.85	0.68-1.06
	3	619	0.75	0.60-0.94
Tics	0	3	1.00	Reference
	1	2	0.17	0.03-1.04
	2	40	1.14	0.35-3.73
	3	25	1.12	0.34-3.77
Ticst	0	0	0.00	Not estimable
	1	2	0.18	0.04-0.76
	2	38	0.98	0.58-1.62
	3	25	1.00	Reference
ADD	0	15	1.00	Reference
	1	34	0.62	0.34-1.14
	2	105	0.49	0.29-0.85
	3	68	0.47	0.27-0.83
Unspecified developmental delay	0	20	1.00	Reference
	1	85	1.20	0.74-1.96
	2	234	0.80	0.51-1.26
	3	146	0.73	0.46-1.16

\* Adjusted for gender, year of birth, and month of birth (general developmental disorders only).  
 † Results from the analysis that excluded those who did not receive 3 doses of DTP/DT by 366 days.

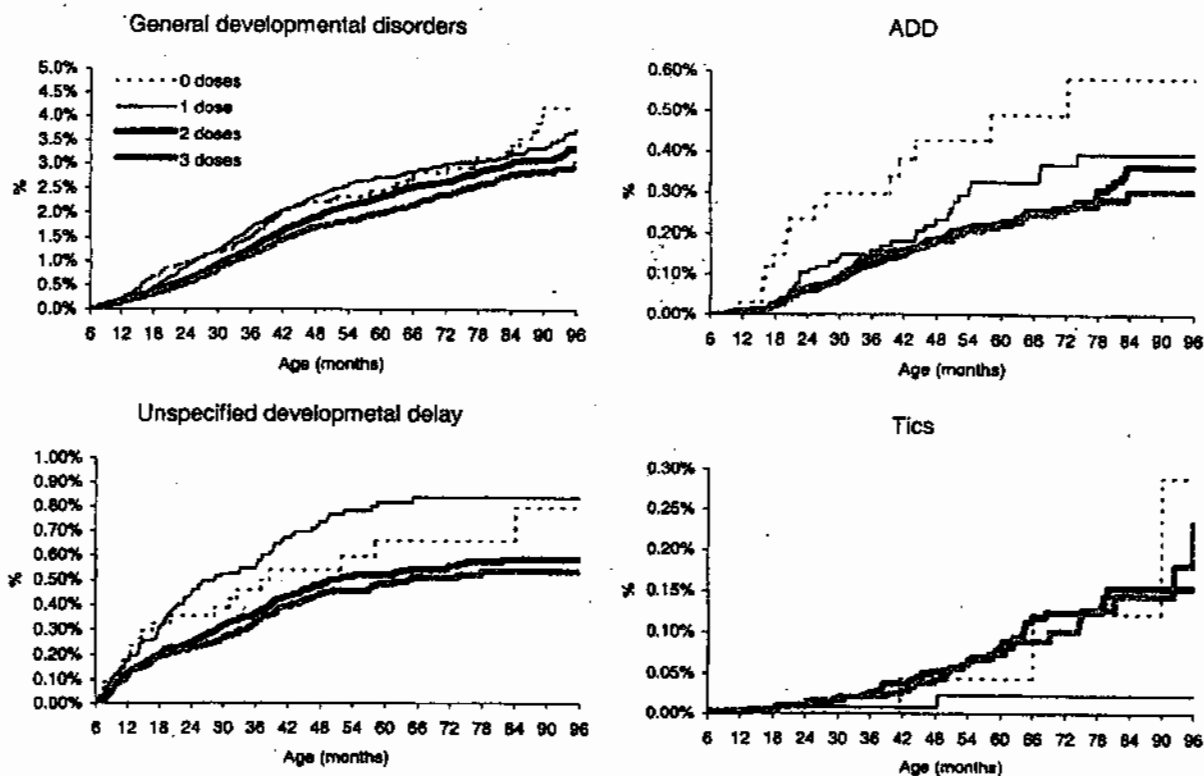


Fig 3. Cumulative percentage of children with general developmental disorders, ADD, unspecified developmental delays, and tics from 6 months to 96 months of age, stratified according to DTP/DT doses received by 4 months of age. Plots are derived from the inverse of the Kaplan-Meier survival curves and take account of variable follow-up times in individuals.

tion were accurate and that the dates of the events recorded in the GPRD were correct or close to the date noted in the GP record.

DISCUSSION

With the possible exception of tics, there was no evidence of an increased risk of various neurodevel-

opmental disorders with increasing thimerosal exposure at a young age via DTP/DT vaccination in the United Kingdom. For general developmental disorders, unspecified developmental delay, and ADD, there was an apparent protective effect from increasing thimerosal exposure. These outcomes all had a median age at first mention at a relatively young age

Case 5:03-cv-00141-TJW Document 328-5 Filed 01/25/2005 Page 30 of 56  
 and therefore were more likely to be affected by confounding factors that are also associated with delayed or incomplete vaccination. Outcome conditions first mentioned when the child was older did not show any evidence of an association with DTP/DT dosage, with the exception of the apparent higher risk of tics in 1 analysis.

Although we were able to make some exclusions on the basis of medical events in the first 6 months of life, a limitation of our study was the inability to adjust for many potential confounding factors, such as unrecorded medical conditions and socioeconomic factors. The longitudinal United Kingdom study, published with this article,<sup>11</sup> did have information available on potential confounding variables. In that study, early thimerosal exposure generally showed no association or was protective. The size of the protective effects reduced when controlling for confounding variables, although the changes were small. This suggests that additional adjustment for confounding in the GPRD study would have a relatively small effect.

Our study has many similarities to the US VSD study and, with the exception of tics, does not confirm the hypotheses raised by the preliminary analysis of that study. Both studies were cohort studies with limited adjustment for confounding. The main difference was the lower total thimerosal exposure in the United Kingdom. It should be noted, however, that the exposure in the United Kingdom by 4 months of age was similar to the United States by the same age; however, in the United States, exposure increased further from 4 to 7 months. If the increased risk in the US study were attributable only to the additional thimerosal exposure after 4 months of age, then it is possible that our study may not have been able to detect the risks found in the US study. In the final analysis of the US cohort study,<sup>13</sup> which had a longer follow-up time and separate analyses for each of the 2 HMOs and also controlled for other variables including health care-seeking behavior, the only variables that remained significant were tics in 1 HMO and language delay in the other. Therefore, many of the preliminary results from the US study were probably attributable to confounding or chance.

The validation exercise confirmed most diagnoses with only 7% of the sample validated deemed incorrectly coded. An additional 13% were questionable because they reflected only parental concern or could not be located in the notes. This lack of specificity is a limitation of the study because it biases against finding an association. If we assume that a conservative 20% of cases have a false diagnosis and that there is a true HR per dose of 1.20, then this bias will result in a slightly lower observed HR of 1.15. Other validation exercises undertaken using the GPRD have found clinical diagnoses to be accurate.<sup>14-16</sup> The predominance of boys as well as the median age at first mention was as expected for the various conditions<sup>17</sup> and provides a degree of validation.

The question remaining is whether there could be a true effect of thimerosal exposure on tics. Evidence supporting a true effect is that it was significant in

the US study and in a secondary analysis in the GPRD study; however, there are many reasons to doubt that there is a true effect. First, the US study was a screening study that looked at many outcomes; the borderline significance in 1 HMO of tics merely raised the question. Second, although the GPRD study gave a borderline significant association, the Avon longitudinal United Kingdom study showed no evidence of a relationship between thimerosal exposure and tics or twitches despite that this outcome was reported for ~150 children. Third, the validation exercise revealed that the vast majority of tics were minor transient events. Finally, no other developmental outcomes were found to be associated with thimerosal exposure, contrary to what would be expected if there were a true effect on tics. Although the possibility of a true effect of thimerosal on minor transient tics cannot be ruled out, it is more plausible that the association found is a chance effect or the result of confounding.

Other than the US VSD study, the only other published cohort study that has assessed exposure to thimerosal-containing vaccines and any of the outcomes that we looked at is a study in Denmark that looked at autism.<sup>18</sup> The thimerosal exposure in this study was 25 µg of Hg at 5 weeks, then 50 µg of Hg at 9 weeks and 10 months. As with our study, the authors found no evidence of an association.

A recent study that measured Hg levels in blood and excretion via the stools and urine in term infants who received vaccines that contained thimerosal<sup>2</sup> found no evidence of a rise in blood concentrations above "safe values" and showed that Hg in ethylmercury is eliminated rapidly via the stools. This provides additional evidence that 3 doses of DTP given at monthly intervals does not present an Hg-related risk for neurodevelopmental disorders.

The results of the 2 United Kingdom studies were presented to the WHO Global Advisory Committee on Vaccine Safety in June 2002.<sup>8</sup> These studies contributed to the conclusion that there is currently no evidence of mercury toxicity in infants, children, or adults who are exposed to thimerosal in vaccines and that there is no reason to change current immunization practices with thimerosal-containing vaccines on grounds of safety. This conclusion is particularly important for developing countries that administer thimerosal-containing DTP vaccines according to the expanded immunization schedule.

#### ACKNOWLEDGMENTS

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We thank Franky Lever for assistance in determining the study feasibility.

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## TWO MINORITIES SPUR RAPID U.S. GROWTH

"Explosive growth among Hispanic and Asian-Americans propelled a surge in the United States population from 2000 to 2003 to nearly 300 million people, the Census Bureau reported on Monday. The number of people of Hispanic descent, the nation's largest minority group, rose to 39.9 million, a 13 percent increase from April 2000 to July 2003, the agency said. That far outpaced the 3 percent increase in the American population during the same time, to 290.8 million. Asian-Americans were the next fastest growing among the large minority groups, up 12.6 percent, to 11.9 million, while the black population rose nearly 4 percent, to 37 million. About 4.3 million people listed themselves as of more than one race, up 10.5 percent from 2000."

Associated Press. *New York Times*. June 15, 2004

Noted by JFL, MD

that are recognized but, instead, the hydrogen peroxide produced when urate is degraded by urate oxidase (uricase). Because mice express urate oxidase, hydrogen peroxide may be the murine "ultimate danger signal." However, some questions remain. Is the effect specific to hydrogen peroxide, or do other reactive oxygen species such as hydroxyl or superoxide suffice? Is there a role for catalase and glutathione peroxidase, which rapidly degrade hydrogen peroxide? Knockout mice lacking xanthine oxidase have been generated (but they are "runted" and die by six weeks of age)<sup>2</sup>; do they lack the ability to respond to danger signals? Can human cells respond to urate crystals? Can such cells respond to reactive oxygen species, or was this pathway sac-

rificed when xanthine oxidase expression was lost? All these questions can be approached experimentally. We look forward to the answers and suspect that further complexity awaits.

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## Autism and DPT Vaccination in the United Kingdom

**TO THE EDITOR:** On the basis of information recorded in the General Practice Research Database in the United Kingdom, we previously reported that the risk of receiving a diagnosis of autism between two and five years of age was four times as high among boys born in 1993 as among boys born in 1988.<sup>1</sup> We presented evidence that this increase had no relation to the use of mumps, measles, and rubella vaccine, a finding similar to that reported by Madsen et al. in the *Journal* in a study based on data from Denmark.<sup>2</sup> We recently reported on a study of 126 cases of autism in boys two to four years of age who were born between 1990 and 1998 and 624 controls (matched for age, sex, general practice, and the index date of the case), in which we found evidence that the increase in the incidence of autism in the United Kingdom was related to changes in diagnostic practices.<sup>3</sup>

Because it has been proposed that the development of autism may be associated with exposure to mercury in vaccines containing the preservative thimerosal, we further analyzed data from our recent case-control study to evaluate the effects of exposure to diphtheria, pertussis, and tetanus (DPT) vaccines, which are the only thimerosal-containing vaccines routinely used in the United Kingdom. Since 1990, it has been recommended that DPT vac-

cination be given at two, three, and four months of age in the United Kingdom.

For this analysis, we excluded 4 of 126 patients with autism (and their controls) and 17 additional controls for whom we could not ascertain the primary DPT vaccination schedule because the child's medical history was not recorded in the General Practice Research Database from the time of birth. Among the remaining 122 patients with autism and 587 controls, 117 patients (96 percent) and 561 controls (96 percent) had three primary DPT vaccinations. Three DPT vaccinations were recorded by six months of age in 112 of the patients (92 percent) and 518 of the controls (88 percent), which was not a significant difference (odds ratio, 1.6; 95 percent confidence interval, 0.7 to 3.3;  $P=0.23$ ). The same proportion of patients (2 percent) as controls (2 percent) received separate component vaccines as their primary immunization (e.g., three diphtheria-tetanus vaccinations and three pertussis vaccinations).

Our results are in close agreement with a separately conducted cohort analysis of data from the General Practice Research Database, recently presented by Elizabeth Miller to the U.S. Institute of Medicine,<sup>4</sup> which showed no evidence of an increased risk of autism or other developmental problems related to exposure to thimerosal in vaccines

CORRESPONDENCE

given to infants in the United Kingdom. Taken together, these findings provide further support for the view that exposure to mercury in vaccines is not the cause of the rising incidence of autism diagnosed in the United Kingdom during the past decade.

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**Editor's note:** The authors report serving as consultants to a law firm representing a vaccine manufacturer in litigation over alleged harm from exposure to vaccines.

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# PEDIATRICS

**Thimerosal and the Occurrence of Autism: Negative Ecological Evidence From Danish Population-Based Data**

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Exh. D-16

## Thimerosal and the Occurrence of Autism: Negative Ecological Evidence From Danish Population-Based Data

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**ABSTRACT.** *Objective.* It has been suggested that thimerosal, a mercury-containing preservative in vaccines, is a risk factor for the development of autism. We examined whether discontinuing the use of thimerosal-containing vaccines in Denmark led to a decrease in the incidence of autism.

*Design.* Analysis of data from the Danish Psychiatric Central Research Register recording all psychiatric admissions since 1971, and all outpatient contacts in psychiatric departments in Denmark since 1995.

*Patients.* All children between 2 and 10 years old who were diagnosed with autism during the period from 1971–2000.

*Outcome Measures.* Annual and age-specific incidence for first day of first recorded admission with a diagnosis of autism in children between 2 and 10 years old.

*Results.* A total of 956 children with a male-to-female ratio of 3.5:1 had been diagnosed with autism during the period from 1971–2000. There was no trend toward an increase in the incidence of autism during that period when thimerosal was used in Denmark, up through 1990. From 1991 until 2000 the incidence increased and continued to rise after the removal of thimerosal from vaccines, including increases among children born after the discontinuation of thimerosal.

*Conclusions.* The discontinuation of thimerosal-containing vaccines in Denmark in 1992 was followed by an increase in the incidence of autism. Our ecological data do not support a correlation between thimerosal-containing vaccines and the incidence of autism. *Pediatrics* 2003; 112:604–606; autism, vaccine, thimerosal, mercury, population, epidemiology.

**ABBREVIATIONS.** ICD-8, *International Classification of Diseases, Eighth Revision*; ICD-10, *International Classification of Diseases, 10th Revision*.

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Reprint requests to (K.M.M.) Danish Epidemiology Science Centre, Department of Epidemiology and Social Medicine, University of Aarhus, 8000 Aarhus, Denmark. E-mail: kmm@dadlnet.dk. PEDIATRICS (ISSN 0031-4005). Copyright © 2003 by the American Academy of Pediatrics.

There has been concern that there may be an association between thimerosal, a vaccine preservative that contains ethyl mercury, and neurodevelopmental outcomes, including autism.<sup>1,2</sup> Findings in the field of methyl mercury have been used to suggest causality. Prenatal exposure to low doses of methyl mercury has been associated with subtle neurodevelopmental abnormalities in some studies,<sup>3,4</sup> and symptoms of autism and methyl mercury intoxication have been claimed to be similar.<sup>1</sup> More research has been requested,<sup>5</sup> and a recent study of the concentrations of mercury after exposure to thimerosal-containing vaccines concluded that thimerosal poses very little risk to full-term infants.<sup>6</sup> In Denmark, thimerosal was used in childhood vaccines from the early 1950s until 1992. The objective of our study was to assess the incidence rates of autism among children between 2 and 10 years old before and after removal of thimerosal from vaccines to see if the discontinuation led to a decrease in the incidence of autism.

### PARTICIPANTS AND METHODS

For this study, the period of use of thimerosal vaccines was limited to 1961 until its discontinuation in March 1992 because information about the diagnosis of autism has only been obtainable from a nationwide computerized registration system, the Danish Psychiatric Central Research Register,<sup>7</sup> since 1969 and only children born in 1961 or later were at risk of developing autism before 10 years of age. Thimerosal was used during 1961–1970 in the diphtheria-tetanus-pertussis vaccines given in 4 doses when the child was 5, 6, 7, and 15 months old, and during 1970–1992 in the whole-cell pertussis vaccines given in 3 doses when the child was 5 weeks, 9 weeks, and 10 months old. The concentrations used in the vaccines from 1961–1970 and from 1970–1992 were 0.01% = 0.1 mg thimerosal, which equals 50 µg ethyl mercury per mL vaccine. The amount of vaccine given was 1 mL, except for the first dose of monocomponent pertussis vaccine where it was only 0.5 mL from 1970–1992. This means that children who followed the full vaccination program during the period 1961–1970 had received a total of 400 µg of thimerosal or 200 µg of ethyl mercury by the age of 15 months and during the period 1970–1992 they had received a total of 250 µg of thimerosal or 125 µg of ethyl mercury at 10 months of age. In March 1992 the last batch of thimerosal-containing vaccine was released and distributed from Statens Serum Institut in Denmark. All vaccinations were given free of charge and acceptance of vaccinations in Denmark has always been very high; from 1979 onward data on vaccination coverage was available and coverage rates of >90% were found (information was obtained from the State Serum Institute). Whether the toxicity of methyl mercury and ethyl mercury is the same remains controversial<sup>8,9</sup> but the recommended safe intake level of methyl mercury is estimated to be 0.1 µg/kg body weight/day by the US Environmental Protection Agency.<sup>10</sup> However, other federal regulatory agencies have recommended slightly higher levels.<sup>9</sup>

Psychiatric inpatient treatment in Denmark has been reported to the Danish Psychiatric Central Research Register since 1969, and since 1995 outpatient activities were registered as well, providing the opportunity to examine long-term trends of the occurrence of autism in a total national population. In Denmark, inpatients refer both to children who stay at the hospital overnight and to children who come to the hospital on a daily basis for evaluation and treatment. The proportion of outpatient to inpatient activities was about 4 to 6 times as many outpatients as inpatients with variations across time and age bands. We obtained information on all children who from the second birthday up to, but not including the 10th birthday were diagnosed with autism in the period from January 1, 1971 to December 31, 2000 in the Danish Psychiatric Central Research Register during which period the register is assumed to be complete. The diagnosis of autism in children <2 years of age was considered uncertain. All individuals in Denmark are assigned a unique personal identification number<sup>11</sup> which is used in all national registers. Admissions to psychiatric hospitals in Denmark are coded using this CPR-number, which eliminates the risk of double-counting of cases. The date of onset was defined as the first day of the first admission leading to a diagnosis of psychosis proto-infantilis (*International Classification of Diseases, Eighth Revision [ICD-8]: 299.00*) or psychosis infantilis posterior (*ICD-8: 299.01*) or from 1994 onward, infantile autism (*International Classification of Diseases, 10th Revision [ICD-10]: F84.0*) or atypical autism (*ICD-10: F84.1*).<sup>12,13</sup>

### Statistics

Incidence rates were calculated for each year 1971–2000 using the age and gender specific number of persons in Denmark as a denominator. For each year and age band, we calculated the incidence as the number of people who at that age band and year was diagnosed with autism for the first time divided by the total number of people alive and living in Denmark at that age band and year.

### RESULTS

A total of 956 children with a male to female ratio of 3.5:1 had been diagnosed with autism during the period 1971–2000. Figure 1 shows the incidence rates according to calendar year and age band. The incidence was stable until 1990 and thereafter it increased in all age groups until 1999. Generally, rates were lower in 2000 than in 1999. Further subdivision by gender had no impact on these results (data not shown). In additional analyses we examined data using inpatients only. This was done to elucidate the contribution of the outpatient registration to the change in incidence. The same trend with an increase

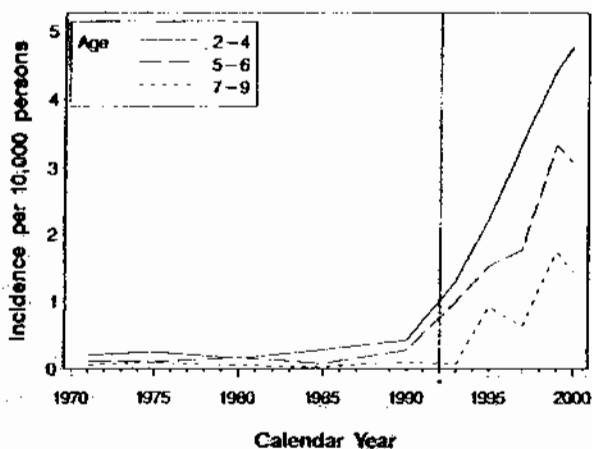


Fig 1. Incidence of autism by age and calendar year. The asterisk (\*) indicates removal of thimerosal-containing vaccines in 1992.

in the incidence rates from 1990 until the end of the study period was seen (data not shown).

There was no trend toward an increase in the incidence of autism during the period when thimerosal was used up to 1990. The incidence of autism began to increase in 1991, but continued to rise after the discontinuation of thimerosal (Fig 1), including increases among children born after 1992 (ie, the peak autism incidence in 1999 among children aged 2 to 4 and 5 to 6 years of age corresponds to children born in 1993–1997 after the introduction of thimerosal-free vaccines).

### DISCUSSION

This study investigated if the discontinuation of thimerosal-containing vaccines paralleled a decrease in the occurrence of autism. The incidence of autism remained fairly constant during the period of use of thimerosal in Denmark, and the rise in incidence beginning in 1991 continued even in the group of children born after the discontinuation of thimerosal. The amount of thimerosal used in vaccines changed during the study period with less amount of thimerosal administered in the period 1970–1992. Moreover, the thimerosal-containing vaccine was gradually phased out meaning that the incidence rates should decline gradually if thimerosal has any impact on the development of autism. However, an increase (rather than a decrease) in the incidence rates of autism was observed.

Only very few incidence studies of autism have been made, and we found similar incidence rates and the same trend of increasing rates of autism in our study compared with studies conducted in other countries.<sup>14,15</sup> The increase in the incidence of autism from 1990 on may be attributable to more attention being drawn to the syndrome of autism and to a change in the diagnostic criteria from the *ICD-8* to the *ICD-10* in 1994. Also, outpatient activities were included in the Danish Psychiatric Central Research Register in 1995 and because many patients with autism in former years have been treated as outpatients this may exaggerate the incidence rates, simply because a number of patients attending the child psychiatric treatment system before 1995 were recorded for the first time, and thereby counted as new cases in the incidence rates.

### CONCLUSIONS

The discontinuation of thimerosal-containing vaccines in Denmark in 1992 was followed by an increase in the incidence of autism. Our ecological data do not support correlation between thimerosal-containing vaccines and the incidence of autism. Our data cannot, of course, exclude the possibility that thimerosal at doses larger than used in Denmark may lead to neurodevelopmental damage.

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#### FAT BABIES AND HEALTH

"In a recent issue an English contemporary calls attention to the mischief that is being done by the present standard that is accepted as regards healthy babies. As this paper well says, at baby shows the prize is practically always given to the fattest baby. There is a tradition current among mothers, as far as the memory of man runneth, that fat babies are just the pink of perfection. The surest index of this is that all manufacturers of artificial infant food advertise their wondrous virtues by photographs of thoroughly rounded, and at times positively obese dumplings of babies. Mothers are very proud of their young hopefuls if they are a mass of curves and dimples with deep folds at all the joints and cushions of fat that conceal their anatomy so effectively as to make them formless little masses of humanity."

*JAMA 100 years ago. JAMA. 2003;289:1866*

*Editor's Note: Not much change in 100 years! Will we ever win this one?*

Noted by JFL, MD

**Thimerosal and the Occurrence of Autism: Negative Ecological Evidence From Danish Population-Based Data**

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## Autism and Thimerosal-Containing Vaccines Lack of Consistent Evidence for an Association

Paul Stehr-Green, DrPH, MPH, Peet Tull, Michael Stellfeld, MD, Preben-Bo Mortenson, DrMedSC,  
Diane Simpson, MD, PhD

- Background:** In 1999, concerns were raised that vaccines containing the preservative Thimerosal™ might increase the risk of autism and/or other neurodevelopmental disorders.
- Methods:** Between the mid-1980s through the late-1990s, we compared the prevalence/incidence of autism in California, Sweden, and Denmark with average exposures to Thimerosal-containing vaccines. Graphic ecologic analyses were used to examine population-based data from the United States (national immunization coverage surveys and counts of children diagnosed with autism-like disorders seeking special education services in California); Sweden (national inpatient data on autism cases, national vaccination coverage levels, and information on use of all vaccines and vaccine-specific amounts of Thimerosal); and Denmark (national registry of inpatient/outpatient-diagnosed autism cases, national vaccination coverage levels, and information on use of all vaccines and vaccine-specific amounts of Thimerosal).
- Results:** In all three countries, the incidence and prevalence of autism-like disorders began to rise in the 1985–1989 period, and the rate of increase accelerated in the early 1990s. However, in contrast to the situation in the United States, where the average Thimerosal dose from vaccines increased throughout the 1990s, Thimerosal exposures from vaccines in both Sweden and Denmark—already low throughout the 1970s and 1980s—began to decrease in the late 1980s and were eliminated in the early 1990s.
- Conclusions:** The body of existing data, including the ecologic data presented herein, is not consistent with the hypothesis that increased exposure to Thimerosal-containing vaccines is responsible for the apparent increase in the rates of autism in young children being observed worldwide. (Am J Prev Med 2003;25(2):101–106) © 2003 American Journal of Preventive Medicine

### Introduction

In June of 1999, concerns were raised that children vaccinated with products containing the preservative Thimerosal™ could receive doses of organic mercury (specifically, the thiosalicylate salt of ethylmercury) that exceeded existing guidelines for intake of methylmercury.<sup>1</sup> These concerns were based on extrapolations from the known effects of prenatal methylmercury exposure.<sup>2</sup> Because there are limited data on the

toxicology and pharmacokinetics of Thimerosal and ethylmercury, for the purpose of these extrapolations it was assumed that many features of the toxicity of ethylmercury were qualitatively similar to those of methylmercury.<sup>1</sup>

It was subsequently suggested that the apparent increase in the incidence of autism in the United States in the 1990s occurred at about the same time that *Haemophilus influenzae* b (Hib) and hepatitis B (hep B) vaccines were first universally recommended (i.e., in 1990 and 1991, respectively), thereby increasing the average cumulative exposure to Thimerosal from vaccines administered to infants. Prior to that time, the only sources of Thimerosal from vaccines on the recommended childhood immunization schedule were diphtheria–tetanus–pertussis (DTP) (later replaced by diphtheria–tetanus–acellular pertussis [DTaP]) and diphtheria–tetanus (DT) vaccines. Although the maximum theoretical dose of Thimerosal from vaccines varied depending on the brand and combination vaccines used, most children in the United States who received the four universally recommended doses of

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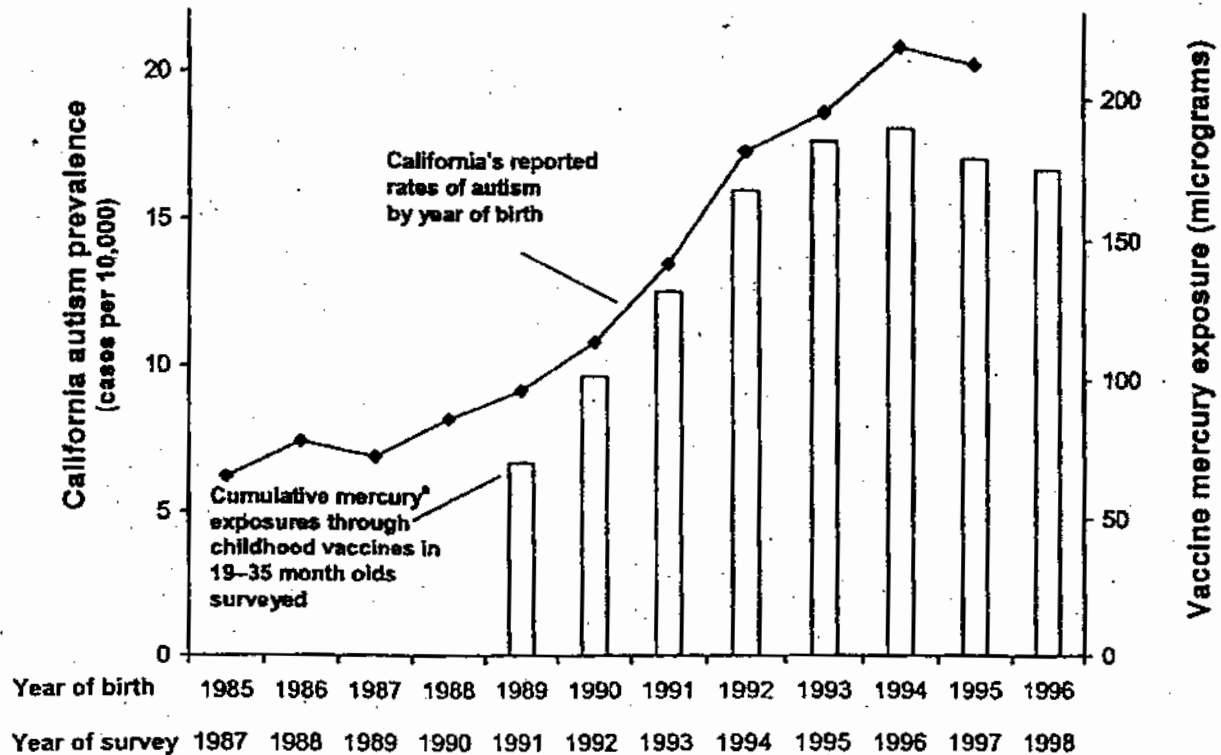


Figure 1. Graphical ecologic analysis presented by Blaxill<sup>3</sup> to the Institute of Medicine on July 16, 2001, comparing the estimated average cumulative dose of mercury exposure in the United States from vaccines, and the estimated prevalence (per 10,000 population) of children diagnosed with autism-like disorders seeking special education services for autism in California from 1987 to 1998, by birth-year cohort.

\*Includes DPT, *Haemophilus influenzae* B, and hepatitis B exposures weighted by survey year compliance.

DTaP/DTP/DT, four doses of Hib, and three doses of hep B in 1999 would have received a 237.5  $\mu$ g cumulative dose of ethylmercury by age 2 years.

In July 2001, the Institute of Medicine (IOM) Immunization Safety Review Committee held a public meeting to review data and testimony regarding the alleged association of neurodevelopmental effects (including autism) and Thimerosal-containing vaccines. At this meeting, Blaxill<sup>3</sup> presented an ecologic analysis comparing the estimated average cumulative dose of mercury exposure (i.e., the average ethylmercury dose, calculated by multiplying the amount of Thimerosal in the various vaccines by the vaccine-specific coverage rate for U.S. children aged 19 to 35 months, by birth year cohort) to the estimated prevalence of autism in children in California per 10,000 population, by birth year. The prevalence of autism was defined as occurrence of persons with autism or other pervasive developmental disorders (PDD), based on an individualized client development evaluation performed at intake into the California Department of Developmental Services regional and developmental center system during 1987-1998 and coded as International Classification of Diseases (ICD)-9 codes 299.1, 299.80, or 299.88<sup>4</sup>; or

(1) "Autism, full syndrome" (no ICD-9 code specified); (2) "Autism, residual state" (no ICD-9 code specified); or (3) "Autism suspected, not diagnosed" (no ICD-9 code specified).<sup>5</sup> The graphical presentation of these data (Figure 1) showed that the number of children in California coded as having autism-like disorders seeking special education services per 10,000 population remained reasonably constant through the mid-1980s, began to rise slightly in 1988, and then began to rise more dramatically in 1990.

As with most ecologic analyses, these data had several limitations. Nonetheless, because of the high level of public interest and the potentially important public health implications, collection of additional ecologic data to further examine this alleged association was performed. In conducting this investigation, we consulted with public health officials and researchers in Sweden and Denmark; both countries have historically maintained high-quality records on vaccine components, recommended vaccination schedules, population vaccination coverage rates, and the occurrence of autism-like disorders.

## Methods

In Sweden, data were collected at the national level on cases of autism (defined as "infantile autism, including atypical autism" [ICD-9 codes 299.x for 1987-1997 and ICD-10 codes F84.x for 1997-1999]) diagnosed in inpatient settings among 2 to 10 year olds during from 1987 to 1999. Data collection also included vaccination coverage levels dating back to 1980 as well as administrative information from the Swedish Institute for Infectious Disease Control for the time period(s) of use and vaccine-specific amounts of Thimerosal for all vaccines used in Sweden.

For each birth-year cohort, the average cumulative dose of ethylmercury from vaccines was estimated by multiplying the amount of ethylmercury in Thimerosal-containing vaccines used in Sweden by the vaccine-specific coverage rate for Swedish children aged <2 years. The incidence rate of autism was calculated by dividing the number of cases of autism diagnosed among 2- to 10-year-old inpatients during 1987-1999 by the total number of person-years accumulated during that time period for each annual cohort of children born between 1980 and 1996 (multiplied by 100,000 person-years). Using these data, the ecologic association of the birth-year, cohort-specific administration of Thimerosal-containing vaccines, and the incidence of autism requiring hospitalization among children born in Sweden from 1980 to 1996 was examined.

In Denmark, we examined data on incident cases of autism diagnosed in both inpatient and outpatient settings. The data were from a national registry of children with neurological disorders and compiled by researchers at the Danish National Centre for Register-Based Research. This registry included children who had been admitted to a psychiatric hospital or received outpatient care prior to 1994 with a diagnosis of "psychosis proto-infantilis" (ICD-8 code 299.00); "psychosis infantilis posterior" (ICD-8 code 299.01); or, from 1994 onward, "infantile autism" (ICD-10 code F84.0) or "atypical autism" (ICD-10 code F84.1). Data were also collected at the national level on vaccination coverage levels dating back to 1981, in addition to administrative information from the Danish Statens Serum Institut for the time period(s) of use and vaccine-specific amounts of Thimerosal for all vaccines used in Denmark.

The average cumulative dose of ethylmercury from vaccines for each birth-year cohort was estimated by multiplying the amount of ethylmercury in Thimerosal-containing vaccines used in Denmark by the vaccine-specific coverage rate for Danish children aged <10 months. The number of autism cases diagnosed among 2 to 10 year olds was totaled for each year between 1983 and 2000. Using these data, the ecologic association of the birth-year cohort-specific administration of Thimerosal-containing vaccines and the annual number of cases of autism diagnosed between 1983 and 2000 among children aged 2 to 10 years in Denmark was examined.

## Results

As shown in Figure 2, the incidence of autism diagnosed among Swedish inpatients aged 2 to 10 years old began to increase in the mid to late 1980s, rising from a rate of 5 to 6 inpatient-diagnosed cases per 100,000

person-years before 1985 to a peak rate of 9.2/100,000 in 1993. This was generally similar to the above-described trend in California during the same time period. Vaccination coverage has remained high in Sweden (i.e., almost always >95% for all age-specific antigens) since 1980, but the use of Thimerosal in vaccines in Sweden decreased and eventually disappeared by 1993. In fact, few vaccines containing Thimerosal were ever used throughout the history of childhood vaccination programs in Sweden. The major exception was the use of Thimerosal-containing DTP (used until 1979) and DT vaccines (used until 1992), both of which contained Thimerosal at a concentration of 0.01% (i.e., identical to the amount of Thimerosal contained in DTP and DT vaccines used in the United States). A very small number of children also received Thimerosal-containing single-antigen Hib vaccine and/or acellular pertussis vaccines used in a clinical trial prior to 1992. However, since 1992, Thimerosal has not been used in vaccines administered as part of the routine childhood vaccination program in Sweden, except for the very small number of children born to high-risk mothers (<1% of the annual birth cohort) who may have received Thimerosal-containing hep B. Thus, most children in Sweden who received the three recommended doses of Thimerosal-containing DTP/DT prior to 1992 would have received a 75- $\mu$ g cumulative dose of ethylmercury by age 2 years.

As shown in Figure 3, the experience in Denmark was similar to that in Sweden, where the annual number of autism cases rose from <10 cases among 2 to 10 year olds before 1990 to a peak of 181 cases in 1999. This increase, which began around 1990, affected all age groups aged >2 years and resulted in an estimated prevalence of 8.1 cases per 10,000 persons at the end of 2000.<sup>6</sup> As in Sweden, vaccination coverage in Denmark has remained high (i.e., almost always  $\geq$ 90% for all age-specific antigens) since 1980. In Denmark, throughout the period between 1970 and 1989, Thimerosal was used only in whole-cell pertussis (wP)-containing vaccines at a concentration of 0.01% (i.e., identical to the amount of Thimerosal in DT and pertussis-containing vaccines in the United States and Sweden). Therefore, children in Denmark who received the three recommended doses of Thimerosal-containing wP between 1970 and 1991 would have received a 125- $\mu$ g cumulative dose of ethylmercury by age 10 months. In April 1992, the last batch of Thimerosal-containing wP vaccine was produced in Denmark, and its use was eliminated entirely by the end of 1992. Consequently, the proportion of children who received a 125- $\mu$ g cumulative dose of ethylmercury by age 10 months decreased dramatically between 1991 and 1993. Thus, the apparent rise in diagnosed autism cases in Denmark, as in Sweden, occurred during a time of decreasing use (and eventual elimination) of Thimerosal-containing vaccines.

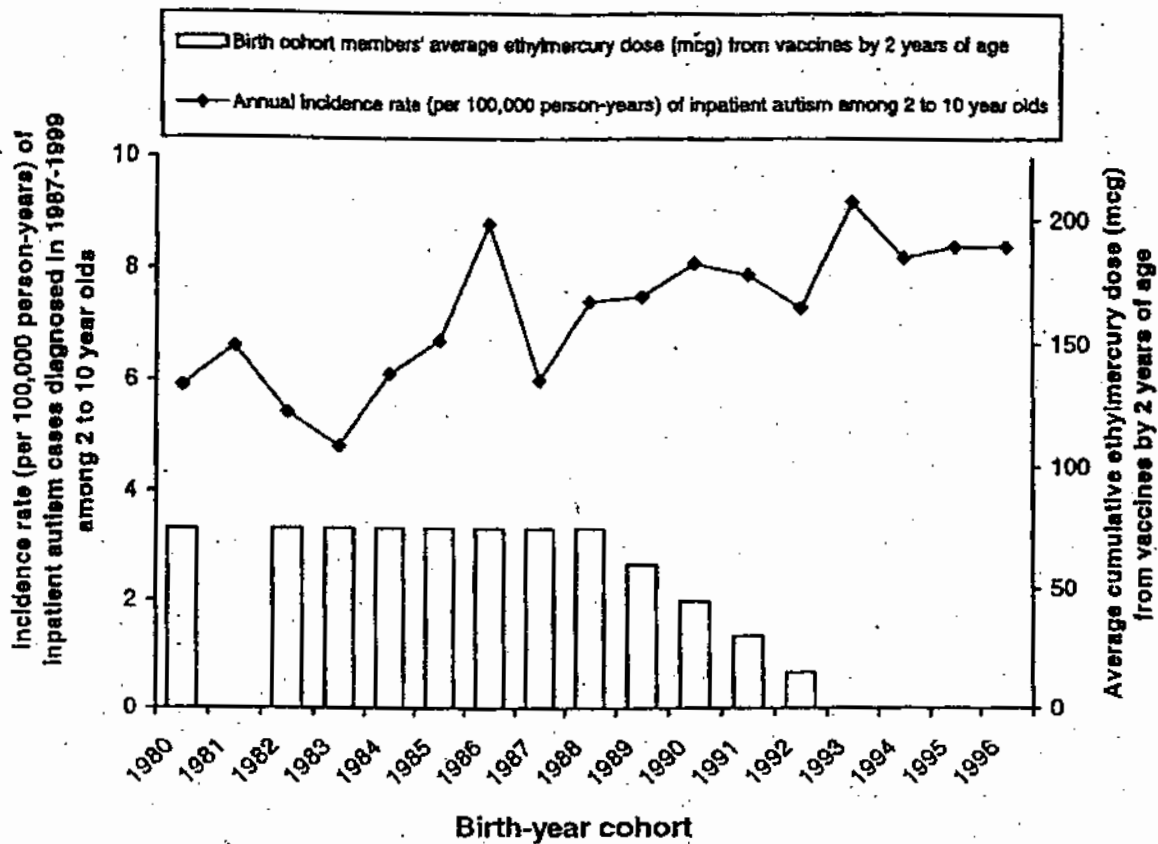


Figure 2. Graphical ecologic analysis comparing average cumulative ethylmercury dose received from vaccines and the incidence rate (per 100,000 person-years) of autism cases in children aged 2 to 10 years diagnosed during 1987-1999 in inpatient settings in Sweden, by birth-year cohort from 1980 to 1996. (Data not available for year 1981.)

### Discussion

At first glance, since the increasing vaccination coverage levels in the United States in the early 1990s likely reflect an increasing average exposure to Thimerosal from those vaccines, the results of the ecologic analysis presented to the IOM in July 2001, which showed proximate increases in autism incidence in California, could be argued to be generally consistent with the existence of an etiologic association. On closer examination, however, the upward trend in the prevalence of autism in California (and elsewhere in the United States) appears to have started, albeit at a more moderate rate, in the late 1980s—before the increase in vaccination coverage rates and/or the introduction of additional Thimerosal-containing vaccines (i.e., Hib and hep B) in the early 1990s. Similarly, the rate of autism in Sweden also appears to have begun to increase in the mid to late 1980s and, in fact, may have started much earlier. Population-based data representative of the city of Gothenburg (Sweden's second-largest city) show an earlier increase in the prevalence of autism and autism-like conditions (excluding Asperger syndrome) from 4.0/10,000 children in 1980, to 7.5/10,000 in 1984, to 11.6/10,000 in 1988.<sup>7-9</sup>

Although the data from California are the most complete data currently available in the United States, the case definition used by the California Department of Developmental Services (described above) is somewhat vague and, therefore, difficult to verify and/or replicate. Furthermore, these data are likely subject to potential biases. For instance, at least some of the increase of reported cases of autism in California may have been stimulated by the growing availability of special education services for affected children during this time period. And even though the data systems in Sweden and Denmark achieve a remarkable level of validity and accuracy, similar confounding influences or biases may be present. For instance, several external events in Denmark, summarized below, may have spuriously increased the apparent number of autism cases.

- Prior to 1992, the data in the national register did not include cases diagnosed in one large clinic in Copenhagen (which accounts for approximately 20% of cases occurring nationwide).
- Prior to 1995, the autism cases reported to the national register reflected only cases diagnosed in inpatient settings.

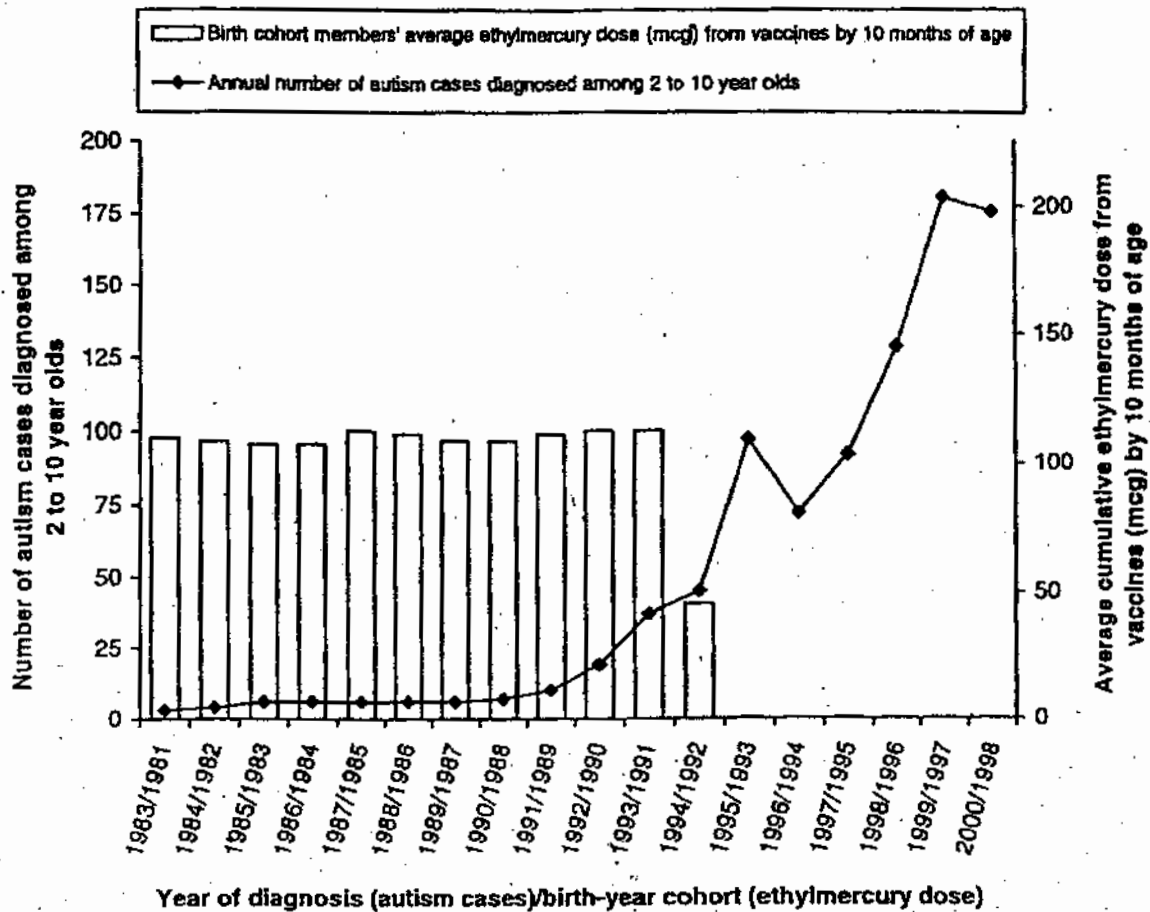


Figure 3. Graphical ecologic analysis comparing the average cumulative ethylmercury dose received from vaccines by birth-year cohort from 1981 to 1998, and the annual number of incident cases of autism in children aged 2 to 10 years diagnosed in Denmark from 1983 to 2000.

- In 1993, when Denmark switched from coding health outcomes using ICD-8 codes to ICD-10 codes, nationwide training seminars for clinicians on the new coding scheme may have stimulated reporting of autism cases (as well as other health outcomes).

Similarly, the data examined from Sweden reflected only cases diagnosed in inpatient settings, for which the data are readily available. Thus, changes over time in the rates of diagnosis of autism-like disorders in inpatient versus outpatient settings may have affected the ascertainment of cases, and differences in the distribution of the setting in which diagnoses have occurred may have affected the comparability of these results over time and among these three countries.

The apparent increase in diagnosed cases of autism may also be due, at least in part, to changes that have occurred over time in diagnostic criteria and increasing professional and public awareness of autism and related disorders. In fact, the diagnostic criteria for Asperger syndrome, Rett syndrome, and childhood

disintegrative disorder were introduced for the first time in 1994 as subcategories of PDD.<sup>4</sup> Of note, these subcategories of PDD accounted for the largest increases in the reported California cases reflected in the data used in the ecologic analysis presented to the IOM.

Finally, at least some of the apparent discrepancy between the California findings and those in Sweden and Denmark are likely the result of the well-known shortcomings of ecologic data rather than a reflection of actual differences in the etiologic process of autism in these respective countries. Ecologic analyses, such as those presented herein, represent empirical investigations involving groups (as opposed to individual persons) as the unit of analysis. Such studies can be useful in exploring possible associations, as well as in searching for areas of possible further study, and are relatively easy to do since group-level data are often more readily available. However, the greatest difficulty in interpreting ecologic studies is that of adequately controlling confounding factors due to unavailability of data

and/or methodologic limitations.<sup>10</sup> Given the ecologic nature of the analyses presented herein and the lack of available detailed data, we were unable to investigate other aspects of this alleged association (e.g., the specific timing of exposure and/or the onset of autism, the existence/nature of a lag time between exposure and disease onset, or the role of genetic predisposition or other co-factors) or the potential influence of confounding factors.

Nonetheless, even though the observed rise in autism cases in both Sweden and Denmark during a time of decreasing use (and eventual elimination) of Thimerosal-containing vaccines in the early 1990s was based on ecologic evidence (and is, therefore, subject to the aforementioned limitations), these results provide compelling evidence in sharp contrast to the alleged association observed in California, during the same time period, which was based on similar ecologic data. More robust studies are currently being planned at the Centers for Disease Control and Prevention and elsewhere to examine the possible association of Thimerosal-containing vaccines and neurodevelopmental problems (including autism) that will be designed to eliminate (or at least mitigate) these limitations (W.C. Thompson, National Immunization Program, Centers for Disease Control and Prevention, personal communication, 2002).

### Conclusion

After considering all the existing evidence, in September 2001 the IOM concluded that "the evidence is inadequate to accept or reject a causal relationship between exposure to Thimerosal from vaccines and . . . autism . . . [However,] . . . the hypothesis is biologically plausible."<sup>11</sup> The authors of the IOM study found no consistent ecologic evidence linking the administration of Thimerosal-containing vaccines with an increasing incidence/prevalence of autism cases. Therefore, it is reasonable to conclude that the body of existing data, including the ecologic data presented herein, are not consistent with the hypothesis that increased exposure to Thimerosal-containing vaccines are responsible for the apparent increases in the rates of autism in young children being observed worldwide. Rather, it seems

more plausible that other factors are affecting these changes, such as those mentioned above: an increased recognition of the disorder in the most and least developmentally delayed children (i.e., compared to children with IQs in the 50 to 70 range) and/or possibly other as-yet-unidentified environmental or genetic factors.

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Financial support for the compilation of the data used in this investigation and the preparation of this report was provided by the National Immunization Program, Centers for Disease Control and Prevention. We are grateful to Victoria Romanus of the Swedish Institute for Infectious Disease Control, Ingrid Trolin of the Swedish Medical Products Agency, Anne-Marie Plesner and Peter Andersen of the Danish Statens Serum Institut, and Roger Bernier and Susan Chu of the Centers for Disease Control and Prevention for their contributions in the design and conduct of this investigation, and in the preparation and review of this manuscript.

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Jill James, PhD

**Evidence for Increased Oxidative Stress and Impaired Methylation in Children with Autism: Metabolic Biomarkers and Genetic Predisposition**

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**Overview**

A little Basic Biochemistry: Folate/Methionine/Glutathione

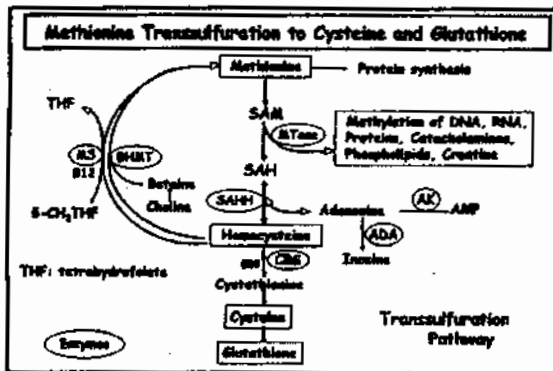
What is Glutathione and Why is it Important?

What is Oxidative Stress and How Does it Damage Cells?

Abnormal Methylation and Oxidative Stress in Autistic Children: Results of an Intervention Trial with Methyl B-12, Folic Acid, and TMS

Increased Frequency of Selected Genetic Polymorphisms Associated with the Metabolic Profile in Children with Autism

Implications of Oxidative Stress and Methylation Deficit in the Pathogenesis of Autism



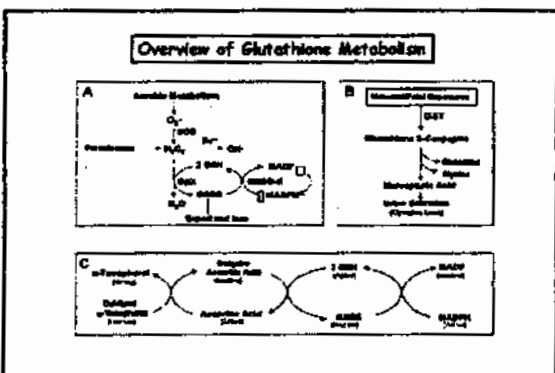
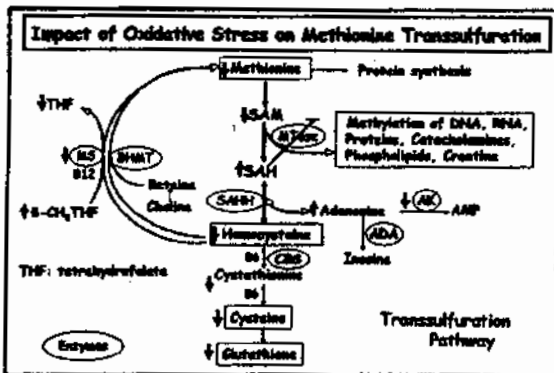
**RELEVANT BACKGROUND INFORMATION**

Methionine is an essential AA: Methionine cycle conserves methionine

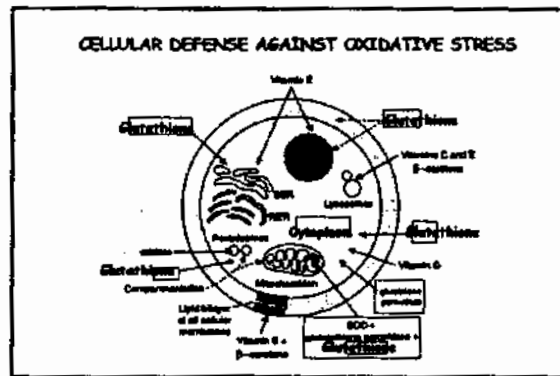
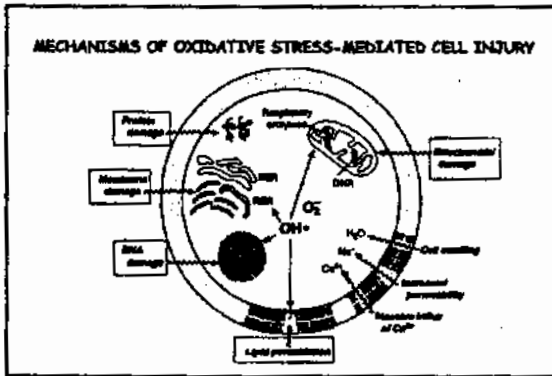
Cysteine is a "conditionally" essential AA: ~50% of cysteine is derived from methionine via the transsulfuration pathway; normal cysteine levels depend on adequate availability of methionine

Cysteine is the rate-limiting amine acid for glutathione (Glu-Gy-Cys) synthesis: the thiol (-SH) group of cysteine is the active antioxidant

Females have higher MS and MAT activity and higher GSH levels than males (faster turnover of methionine cycle) and higher methylation and antioxidant potential: implication for skewed gender ratio in autism?



Jill James, PhD



**Additional Cellular Functions of Glutathione**

**Major Route of Mercury Excretion:** Glutathione/cysteine conjugates excreted in urine and in bile

**Protein Disulfide Status:** Prevents oxidation of cysteine residues in proteins and functional inactivation of proteins

**ATP Production and Mitochondrial Integrity:** Protects mitochondrial membrane integrity

**Integrity of Gut Epithelium:** GSH depletion associated with degeneration mucosal villi and increased gut permeability

**Normal T Cell Subsets and Immune Function:** Normal T cell maturation in the thymus

**Neurotoxicity of Thimerosal in Human Brain Cells is Associated with Glutathione Depletion:**

**Protective Effect of N-Acetyl Cysteine or Glutathione**

S. Jill James, William Slikker, Elizabeth New, Stefanie Jernigan, Stepan Melnyk

Recently accepted to Neurotoxicology for publication this fall!

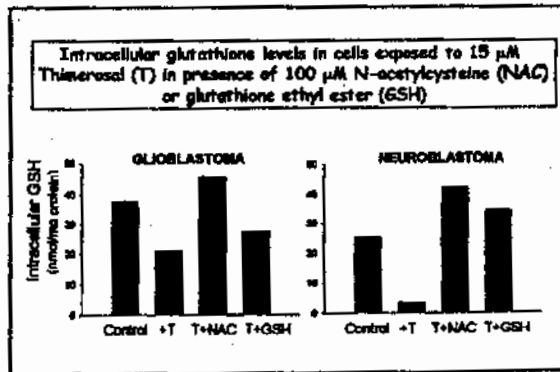
**WORKING HYPOTHESES**

The neurotoxicity of Thimerosal is associated with depletion of glutathione, the major intracellular antioxidant

Ethyl mercury in Thimerosal binds to cysteine thiol (-SH) groups on intracellular proteins and inactivates function.

The cysteine-SH group of glutathione, binds mercury and protects essential proteins from functional inactivation.

Glutathione is the major mechanism of mercury excretion

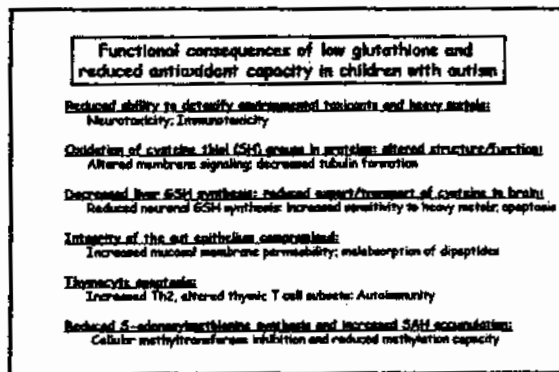
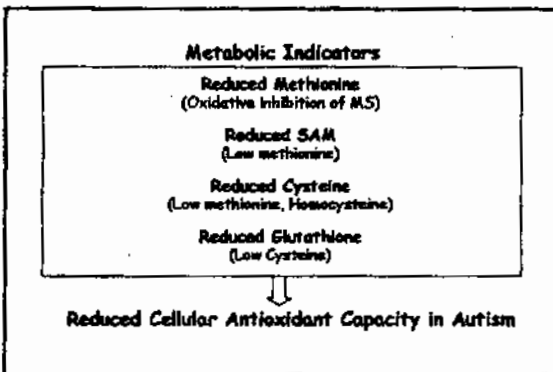
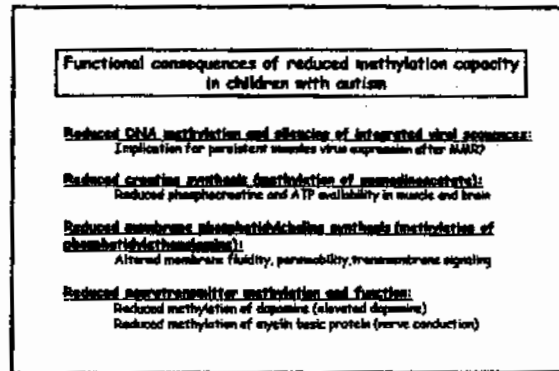
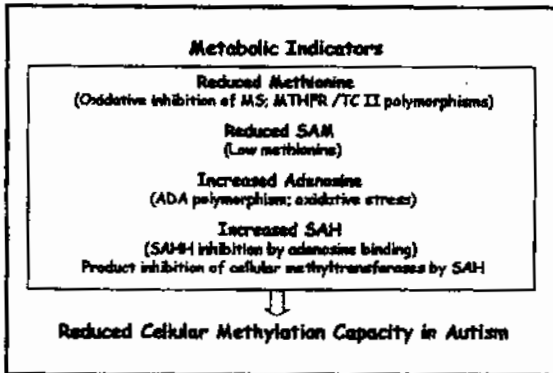






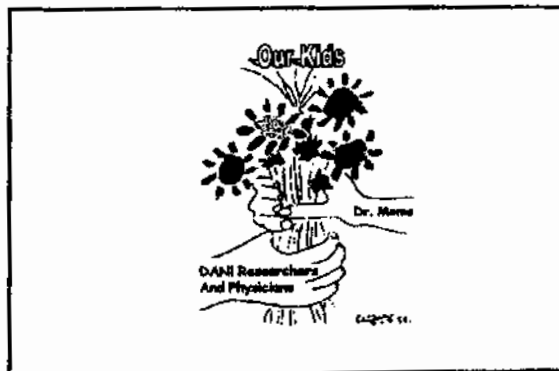


Jill James, PhD



**OVERALL CONCLUSION**

The abnormal metabolic profile in children with autism is consistent with the abnormal genetic profile and strengthens the hypothesis that a genetic susceptibility to oxidative stress and reduced methylation capacity may predispose these children to the neurologic, immunologic, and gastrointestinal dysfunction that occurs with autism.



# A Case-Control Study of Mercury Burden in Children with Autistic Spectrum Disorders

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Jerold J. Kartzinol, M.D.  
James B. Adams, Ph.D.  
Mark R. Geier, M.D., Ph.D.

## ABSTRACT

Large autism epidemics have recently been reported in the United States and the United Kingdom. Emerging epidemiologic evidence and biologic plausibility suggest an association between autistic spectrum disorders and mercury exposure.

This study compares mercury excretion after a three-day treatment with an oral chelating agent, meso-2,3-dimercaptosuccinic acid (DMSA), in children with autistic spectrum disorders and a matched control population. Overall, urinary mercury concentrations were significantly higher in 221 children with autistic spectrum disorders than in 18 normal controls (Relative Increase (RI) = 3.15;  $P < 0.0002$ ). Additionally, vaccinated cases showed a significantly higher urinary mercury concentration than did vaccinated controls (RI = 5.94;  $P < 0.005$ ). Similar urinary mercury concentrations were observed among matched vaccinated and unvaccinated controls, and no association was found between urinary cadmium or lead concentrations and autistic spectrum disorders.

The observed urinary concentrations of mercury could plausibly have resulted from thimerosal in childhood vaccines, although other environmental sources and thimerosal in Rh<sub>0</sub>(D) immune globulin administered to mothers may be contributory.

Regardless of the mechanism by which children with autistic spectrum disorders have high urinary mercury concentrations, the DMSA treatment described in this study might be useful to diagnose their present burden of mercury.

**KEY WORDS:** autism, autistic spectrum disorders, chelation, DMSA, mercury, thimerosal

## Background

Recent studies have analyzed the prevalence of autism from the mid-1980s through 2002 in the United States and the United Kingdom.<sup>1-4</sup> The prevalence of autism is estimated to have risen from one in about 2,500 children in the mid-1980s to as common as one in 150 by 2002. Further, since all of these studies find the prevalence of autism in males to be four times that of females, the male prevalence of this disorder exceeds one in 100. These studies show that the rise in the prevalence in autism is genuine and not the result of population migration, differences in diagnostic criteria, or other potential confounders.

In 2001, the Institute of Medicine (IOM) of the United States National Academy of Sciences<sup>5</sup> determined that a link between mercury from thimerosal contained in childhood vaccines and the recent dramatic increase in neurodevelopment disorders is biologically plausible. Recent studies demonstrate a strong epidemiologic

link between exposure to mercury from thimerosal contained in childhood vaccines and neurodevelopment disorders.<sup>2,4</sup>

The purpose of this study was to evaluate the concentration of mercury in the urine following a three-day treatment with an oral chelating agent, meso-2,3-dimercaptosuccinic acid (DMSA), in children with autistic spectrum disorders in comparison to a control population. Forman et al.<sup>6</sup> have reported on the use of oral treatment with DMSA in children exposed to metallic mercury. The authors found that oral chelation with DMSA produced a significant mercury diuresis in these children. They observed no adverse side effects of treatment. The authors concluded that DMSA appears to be an effective and safe chelating agent for treatment of pediatric overexposure to metallic mercury. In addition, extensive literature supports its safety in the chelation of lead from exposed children.

## Methods

This study is a retrospective analysis of 221 consecutive children with previously established autism spectrum disorders referred and admitted to the International Child Development Resource Center (ICDRC). Each child had been diagnosed with autism (DSM-IV 299.00) or pervasive developmental disorder (DSM-IV 299.80) by outside physicians. A control population of 18 children was also identified without autism spectrum disorders in themselves or among their siblings or their first-degree family members. These healthy children presented to the ICDRC for elective determination of their levels of environmental mercury exposure at the request of their families, and are included here for case comparison. The Arizona State University Institutional Review Board approved our retrospective examination of cases and controls in this study.

All children were examined to exclude those who had dental amalgams. Among the 221 cases, all had received their full scheduled childhood immunizations appropriate for their respective ages. Among the 18 controls, 10 children had received their full childhood immunization schedules, and 8 children had received no childhood immunizations because of religious objections.

Informed consent was obtained from both cases and controls for DMSA chelation treatment. Controls and cases were both challenged with a three-day oral treatment of DMSA (10 mg/kg per dose given three times daily). After the ninth dose, the first voided morning urine was collected (when possible), or an overnight urine collection bag was worn. All laboratory analyses were performed by the Doctors' Data, Inc., in Chicago, Ill. The response to DMSA was measured as micrograms of mercury per gram of creatinine using inductively coupled mass spectrometry, and creatinine was measured using the Jaffe method. The laboratory was not informed whether the specimens were from cases or controls.

In addition to the overall excretion data, several epidemiologic case-control studies were conducted using the available populations. First, it was possible to match 88 cases against 16 controls for age (within one year) and sex, and overall post-DMSA urinary

mercury concentrations were determined. Second, it was possible to match 55 cases against 8 vaccinated controls for age, sex, and vaccine status, and overall post-DMSA urinary mercury, cadmium, and lead concentrations were determined. Finally, as epidemiologic controls, it was possible to match 5 each of vaccinated and unvaccinated controls for age and sex, and overall post-DMSA urinary mercury, cadmium, and lead concentrations were determined.

The statistical package contained in Excel<sup>®</sup> and SISA<sup>®</sup> was employed in this study. We determined means, relative increase (RI) in mean heavy metal excretion in cases compared with controls (mean<sub>cases</sub>/mean<sub>controls</sub>), standard deviation, and statistical significance using a t-test. Our null hypothesis was that the populations under study should have similar distributions of excreted heavy metals, and we accepted a double-sided P-value of <0.05 as statistically significant.

Population Type	Number of Boys	Number of Girls	Mean Age in Years (Range)	Mean Urinary Mercury (mcg/g creatinine) (Range)
Cases	183	38	6.25 (3 to 15)	4.06 ± 8.59 (0 to 58.65)
Controls	14	4	8.85 (3 to 16)	1.29 ± 1.54 (0 to 6.2)

Table 1. Summary of 221 Cases and 18 Controls

**Results**

Table 1 summarizes the number of males and females, mean age in years, and average mcg Hg/g creatinine after DMSA treatment among our 221 cases and 18 controls. Among our 221 cases the boy:girl ratio was 4.88:1, and among our 18 controls the boy:girl ratio was 4:1. Urinary mercury concentrations were significantly higher in cases than in controls (RI)=3.15; P < 0.0002; 95% CI: 1.43 to 4.11).

In the first part of our case-control analysis, we determined the mean and standard deviation of the concentrations of urinary mercury in the 88 cases (5.45 ± 10.9 mcg Hg/g creatinine) and 16 age and sex-matched controls (1.45 ± 1.57 mcg Hg/g creatinine) after DMSA treatment. The urinary mercury concentrations were significantly higher in cases than in controls (RI)=3.76; P < 0.002; 95% CI: 1.60 to 6.41).

The results of the second part of our case-control analyses are summarized in Table 2. We determined the mean and standard deviation of the urinary mercury concentrations in the 55 cases (6.42 ± 12.69 mcg Hg/g creatinine) and 8 age, sex, and vaccine-

	Heavy Metal Examined	Population Examined	Heavy Metal Level (mcg/g creatinine)
	Mercury	55 Cases	6.42 ± 12.69
	Mercury	8 Controls	1.08 ± 1.12
Statistical Assessment			Relative Increase = 5.9 P < 0.005 95% CI: 1.90 to 8.79
	Cadmium	55 Cases	0.48 ± 0.42
	Cadmium	8 Controls	0.36 ± 0.22
Statistical Assessment			Relative Increase = 1.3 P = 0.35 Not Significant
	Lead	55 Cases	18.2 ± 43.3
	Lead	8 Controls	11.8 ± 8.6
Statistical Assessment			Relative Increase = 1.5 P = 0.34 Not Significant

Table 2. Matched Cases and Controls for Heavy Metal Levels Following a 3-Day DMSA Treatment

status-matched controls (1.08 ± 1.12 mcg Hg/g creatinine). We determined that cases had a significantly higher urinary concentrations of mercury after DMSA treatment than did controls (RI)=5.94; P < 0.005; 95% CI: 1.90 to 8.79). As shown in Table 2, both groups had similar urinary concentrations of cadmium and lead after DMSA treatment. Among our age and sex-matched healthy children, we determined that 5 vaccinated controls had similar urinary concentrations of mercury, cadmium, and lead after DMSA treatment compared with 5 unvaccinated controls, as is summarized in Table 3.

	Heavy Metal Examined	Population Examined	Heavy Metal Level (mcg/g creatinine)
	Mercury	5 Vaccinated Controls	0.70 ± 0.71
	Mercury	5 Unvaccinated Controls	1.98 ± 2.40
Statistical Assessment			P = 0.35 Not Significant
	Cadmium	5 Vaccinated Controls	0.42 ± 0.27
	Cadmium	5 Unvaccinated Controls	0.50 ± 0.27
Statistical Assessment			P = 0.65 Not Significant
	Lead	5 Vaccinated Controls	14.0 ± 10.1
	Lead	5 Unvaccinated Controls	16.1 ± 8.5
Statistical Assessment			P = 0.73 Not Significant

Table 3. A summary of a comparison of matched vaccinated and unvaccinated controls for heavy metal levels following a three-day DMSA treatment

**Discussion**

This study shows a strong association between increased urinary mercury concentrations following three days of treatment with DMSA and the presence of an autistic spectrum disorder. The statistically significant association persists when vaccinated cases are compared with matched vaccinated controls. No association was found between post-DMSA urinary cadmium or lead concentrations and autistic spectrum disorders. Lastly, although the study populations were small, the heavy-metal concentrations measured in matched vaccinated and unvaccinated control children were small and showed no statistically significant differences in urinary mercury, cadmium, and lead concentrations following a three-day treatment with DMSA.

Previously, Stajich et al.<sup>11</sup> showed that newborn infants had significant (P < 0.01) several-fold increases in the blood concentrations of mercury during the 48 to 72-hour period following immunization with thimerosal-containing childhood vaccines, compared with pre-vaccination levels.

Pichichero et al.<sup>12</sup> examined the concentrations of mercury in the blood, urine, and stool 3 to 28 days following thimerosal-containing vaccines in 40 full-term infants of age 6 months and younger in comparison to 21 control infants receiving thimerosal-free vaccines. The mean mercury doses received by thimerosal-exposed subjects were 45.6 mcg (range 37.5-62.5) for 2-month-old infants and 111.3 mcg (range 87.5-175.0) for 6-month-old infants. Blood mercury concentrations in thimerosal-exposed 2-month-old infants ranged from less than 3.75 to 20.55 nmol/L; in 6-month-old infants, all values were lower than 7.50 nmol/L. Only 15 blood samples from controls contained quantifiable mercury.

Concentrations of mercury were low in the urine after vaccination but were high in the stools of thimerosal-exposed 2-month-old infants (mean 82 ng/g dry weight) and 6-month-old infants (mean 58 ng/g dry weight). The authors estimated that the blood half-life of ethylmercury was 7 days (95% CI 4-10 days). The study was unable to determine the ultimate disposition of most of the mercury with which infants were injected.

Our analysis shows that children who developed autistic spectrum disorders had significantly greater accumulated mercury than controls. Our results are similar to those of the retrospective study by Holmes et al.<sup>19</sup> They observed that there was a significant relationship between increasingly severe autism and decreasing mercury levels in first baby haircuts in comparison to normal controls. Our results and those of Holmes et al. probably result from a decreased ability of children with autistic spectrum disorders to excrete mercury, resulting in the retention of potentially toxic mercury levels.

Impaired sulphation is observed in autistic spectrum disorders, and this biochemical deficit, possibly a pre-existing genetic condition, may contribute to the observed mercury accumulation, since the normal mechanism of clearing mercury from the body is thought to involve the binding of mercury compounds to sulfhydryl groups.<sup>20</sup>

Mercury concentrations in the human brain are six times greater than the blood.<sup>21</sup> This stems from the fact that thimerosal contains the ethylmercury radical attached to the sulfur atom of the thiol group of salicylic acid. Generally, mercuric ions bind tightly but reversibly to thiol ligands.<sup>22</sup> It is likely, therefore, that the ethylmercury cation of thimerosal dissociates from the thiosalicylic acid moiety immediately after injection to bind to the surrounding thiol ligands present in great excess in tissue proteins.<sup>23</sup>

The buildup of mercury in the tissues of children is particularly alarming in light of a recent article by Baskin et al.<sup>24</sup> They have examined the toxic effects of micromolar concentrations of thimerosal in cultured human cerebral cortical neurons and in normal human fibroblasts. The results demonstrated that thimerosal in micromolar concentrations induced membrane and DNA damage, and initiated caspase-3 dependent apoptosis in human neurons and fibroblasts. In addition, the authors report that thimerosal toxicity may occur at even lower doses than those utilized in their experiments with longer times of exposure. Another recent study by Makani et al.<sup>25</sup> has also demonstrated high cellular toxicity of thimerosal in low micromolar concentrations in T-cells incubated with thimerosal for 24 hours.

A recent article by Nelson and Bauman<sup>26</sup> stated that the overall clinical picture of mercurism—from any known form, dose, duration, or age of exposure—does not mimic that of autism and that no evidence has yet been brought forward to indicate that children exposed to vaccines containing mercurials have more autism than children with less or no such exposure. However, the National Toxicology Program (NTP) within the U.S. Department of Health and Human Services, an interagency program headquartered at the National Institutes of Health's National Institute of Environmental Health Sciences (NIEHS), reports that clumsiness, speech impairment, and emotional disturbances are commonly observed with both acute and chronic thimerosal exposure. These mercurial symptoms are core to the observed abnormalities in autistic spectrum disorders. This observation is supported by Green et al.,<sup>27</sup> who recently reported that clumsiness is a com-

monly observed comorbidity in Asperger's Syndrome, an autistic spectrum disorder.

The results of our present study, combined with the published observations included above, disagree with the views expressed by Nelson and Bauman and support the hypothesis of Bernard et al.,<sup>28</sup> who have compared the similar biological abnormalities commonly found in autism and the corresponding pathologies arising from mercury exposure. Distinct similarities were found between autism and mercury exposure in their effects upon biochemistry, the immune system, the central nervous system structure, neurochemistry, and neurophysiology.

Another study by Bernard et al.<sup>29</sup> has further examined the relationship between thimerosal and autism. They determined that thimerosal was first added to childhood vaccines in the 1930s, and autism was first described in 1943 among children born in the 1930s, suggesting that autism may indeed be an iatrogenic effect of thimerosal.

In addition, Redwood et al.<sup>30</sup> have reported that mercury exposure from childhood immunization is a cause for concern because exposure to low levels of mercury during critical stages of development has been associated with neurological disorders in children, including attention deficit disorder (ADD), learning difficulties, and speech delays.

Moreover, our findings appear to confirm previously published epidemiologic evidence showing a direct association between increasing mercury from thimerosal-containing childhood vaccines and neurodevelopment disorders in children.<sup>31</sup> These studies showed that there was a two to sixfold, statistically significant increased incidence of neurodevelopment disorders following an additional 75-100 mcg dosage of mercury from thimerosal-containing childhood vaccines in comparison to thimerosal-free childhood vaccines. These studies showed dose-response curves demonstrating a close, statistically significant correlation between increasing mercury doses from childhood vaccines and childhood neurodevelopment disorders.

The results of our analyses suggest that mercury should be removed immediately from all biologic products, and others have reached a similar conclusion. Kravchenko et al.<sup>32</sup> stated, "Thus thimerosal, commonly used as a preservative, has been found not only to render its primary toxic effect, but also [to be] capable of changing the properties of cells. This fact suggests that the use of thimerosal for the preservation of medical biological preparations, especially those intended for children, is inadmissible." Cox and Forsyth<sup>33</sup> reported, "However, individual cases of severe reactions to thimerosal demonstrate a need for vaccines with an alternative preservative." Similarly, "...reactions can be expected in such a high percentage of merthiolate-sensitive persons that merthiolate in vaccines should be replaced by another antibacterial agent."<sup>34</sup> Rohyans et al.<sup>35</sup> revealed in 1984, "Although aqueous merthiolate has been used for years as a topical antiseptic, a recent review of its use by the Food and Drug Administration resulted in its classification as 'less than effective.' Furthermore, two of the ingredients (thimerosal and borate) in merthiolate are toxic if absorbed or injected." In addition, Scal et al.<sup>36</sup> reported in *The Lancet*, "Thimerosal is a weak antibacterial agent that is rapidly broken down to products, including ethylmercury residues, which are neurotoxic. Its role as a preservative in vaccines has been questioned, and the pharmaceutical industry itself considers its use as historical."

## Conclusion

Analysis of post-DMSA urinary mercury excretion found a strong, statistically significant association between greatly increased urinary mercury concentrations and the presence of autistic spectrum disorders in vaccinated children.

The mercury levels measured in this study could plausibly have resulted from exposure to mercury in routine childhood vaccines in the United States, while thimerosal in Rh<sub>0</sub>(D) immune globulin and other potential environmental sources of mercury may be contributory.

Our study is unable to determine whether the statistically significantly higher urinary concentrations of mercury measured in cases in comparison to controls is caused by higher exposure to mercury, reduced ability to excrete mercury, or a combination of these explanations. Regardless of the mechanism by which children with autistic spectrum disorders accumulate high mercury levels, the DMSA treatment course described in this study appears useful and important in determining mercury body burden.

The data from this study, along with emerging epidemiologic data showing a link between increasing mercury doses from childhood vaccines and childhood neurodevelopment disorders, increases the likelihood that mercury is one of the main factors leading to the large increase in the rate of autism and other neurodevelopment disorders. It is to be hoped that removing thimerosal from all childhood vaccines will contribute to a decline in the numbers of new cases of autistic spectrum disorders.

Unfortunately, as discussed in a recent publication,<sup>28</sup> many of the vaccines recommended for the childhood immunization schedule contained the full doses of thimerosal through 2002 (FDA, personal communication), and in addition, pediatric vaccines such as influenza, diphtheria-tetanus (DT), and possibly others, still contain the full amounts of thimerosal in 2003. Therefore, it may be quite some time before a decrease in the prevalence of neurodevelopment disorders is seen.

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Potential conflict of interest: Dr. Mark Geier has been an expert witness and a consultant in cases involving adverse reactions to vaccines before the U.S. Vaccine Compensation Act and in civil litigation. David Geier has been a consultant in cases involving adverse reactions to vaccines before the U.S. Vaccine Compensation Act and in civil litigation.

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## IMMEDIATE COMMUNICATION

# Activation of methionine synthase by insulin-like growth factor-1 and dopamine: a target for neurodevelopmental toxins and thimerosal

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Methylation events play a critical role in the ability of growth factors to promote normal development. Neurodevelopmental toxins, such as ethanol and heavy metals, interrupt growth factor signaling, raising the possibility that they might exert adverse effects on methylation. We found that insulin-like growth factor-1 (IGF-1) and dopamine-stimulated methionine synthase (MS) activity and folate-dependent methylation of phospholipids in SH-SY5Y human neuroblastoma cells, via a PI3-kinase- and MAP-kinase-dependent mechanism. The stimulation of this pathway increased DNA methylation, while its inhibition increased methylation-sensitive gene expression. Ethanol potently interfered with IGF-1 activation of MS and blocked its effect on DNA methylation, whereas it did not inhibit the effects of dopamine. Metal ions potently affected IGF-1 and dopamine-stimulated MS activity, as well as folate-dependent phospholipid methylation: Cu<sup>2+</sup> promoted enzyme activity and methylation, while Cu<sup>+</sup>, Pb<sup>2+</sup>, Hg<sup>2+</sup> and Al<sup>3+</sup> were inhibitory. The ethylmercury-containing preservative thimerosal inhibited both IGF-1- and dopamine-stimulated methylation with an IC<sub>50</sub> of 1 nM and eliminated MS activity. Our findings outline a novel growth factor signaling pathway that regulates MS activity and thereby modulates methylation reactions, including DNA methylation. The potent inhibition of this pathway by ethanol, lead, mercury, aluminum and thimerosal suggests that it may be an important target of neurodevelopmental toxins.

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**Keywords:** autism; attention deficit hyperactivity disorder; PI3-kinase; D4 dopamine receptor; DNA methylation; phospholipid methylation; lead; mercury

## Introduction

Developmental disorders include a spectrum of neurological conditions characterized by deficits in attention, cognition and learning, frequently accompanied by abnormal behaviors. Severe deficits may be recognized at birth, but a failure to achieve standard milestones during initial years of life remains the primary basis of diagnosis in most cases. While the underlying cause(s) remains obscure for many developmental disorders, metabolic abnormalities involving purine synthesis (eg Lesch–Nyhan Syndrome and adenylosuccinate lyase deficiency)<sup>1,2</sup> or impaired methylation-dependent gene silencing and/or imprinting (Rett and Fragile-X syndromes)<sup>3,4</sup> suggest biochemical mechanisms that may be in-

involved. The development disorders can also be caused by exposure to toxins (eg ethanol, in fetal alcohol syndrome; heavy metals, in lead poisoning),<sup>5,6</sup> although the precise mechanisms underlying their toxicity are not known. The recent increase in the incidence of autism has led to the speculation that environmental exposures including vaccine additives (ie aluminum and the ethylmercury-containing preservative thimerosal) might contribute to the triggering of this developmental disorder.<sup>7</sup>

Normal development is closely related to cellular differentiation, and growth factor-initiated signaling promotes differentiation of pluripotent cells.<sup>8</sup> Furthermore, altered patterns of DNA methylation and associated gene silencing underlie phenotypic differences between undifferentiated and differentiated cells.<sup>9</sup> Together, these observations suggest that growth factors promote cellular differentiation by producing effects on DNA methylation. This suggestion is reinforced by the observation that

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blocking methylation interferes with growth factor response.<sup>10,11</sup>

Methylation reactions, including DNA methylation, are generally controlled by the ratio of the methyl donor *S*-adenosylmethionine (SAM) to its demethylated product *S*-adenosylhomocysteine (SAH), since SAH retains considerable affinity for methyltransferase enzymes.<sup>12,13</sup> Methionine synthase (MS) exerts an important influence on the [SAM] to [SAH] ratio by efficiently converting homocysteine to methionine, using 5-methyltetrahydrofolate as the methyl donor. This prohibits the reversion of homocysteine to SAH, which is otherwise thermodynamically favored.<sup>14</sup> In some tissues, but not the brain, homocysteine can also be converted to methionine by a betaine-dependent methyltransferase. Thus methylation reactions in the brain are highly dependent on MS activity.

In addition to the methylation of homocysteine, MS is also essential for folate-dependent methylation of membrane phospholipids carried out by the D4 dopamine receptor.<sup>15-17</sup> Dopamine activation of the D4 receptor initiates a four-step cycle of phospholipid methylation (PLM) in which the side chain of a methionine residue in the receptor is adenosylated, enabling transfer of its methyl group to the head group of an adjacent phospholipid. Following the removal of the adenosyl group by SAH hydrolase, MS provides a new, folate-derived methyl group to the side chain, thereby supporting dopamine-stimulated PLM. In light of studies linking attention-deficit hyperactivity disorder (ADHD) to genetic variants of the D4 receptor found only in primates,<sup>18</sup> we have proposed that dopamine-stimulated PLM might play an important role in attention and in attention-initiated learning.<sup>19</sup>

In the current study, we investigated the ability of dopamine and insulin-like growth factor-1 (IGF-1) to regulate MS activity and folate-dependent PLM in SH-SY5Y human neuroblastoma cells, and found that they stimulated activity via a PI3-kinase- and MAP-kinase-dependent signaling pathway. Furthermore, we examined the ability of several neurodevelopmental toxins to interfere with this novel mode of regulation. Their potent inhibitory effects raise the possibility that impaired MS activity may contribute to developmental disorders and to disorders of attention.

#### Materials and methods

##### Phospholipid methylation

SH-SY5Y cells were grown in six-well plates in  $\alpha$ -MEM supplemented with 10% FBS and 1% penicillin/streptomycin/fungizone. After a wash with Hank's balanced salt solution, cells were incubated for 30 min in 600  $\mu$ l of Hank's solution containing 1  $\mu$ Ci/ml [<sup>14</sup>C]formate (or [<sup>3</sup>H-methyl]methionine), in the presence of IGF-1 or dopamine. Drugs or metal salts were added 30 min prior to the period of radiolabeling. The reaction was terminated by an initial wash with ice-cold unlabeled Hank's solution followed by 500  $\mu$ l

ice-cold 10% TCA. After scraping, cells were sonicated and an aliquot was removed for protein assay. Following centrifugation, the pellet was dispersed in 1.5 ml of 2 N HCl/MeOH/CHCl<sub>3</sub> (1:3:6), vortexed and allowed to separate. The lower CHCl<sub>3</sub> layer was washed twice with 400  $\mu$ l of 0.1 N KCl in 50% MeOH, and an aliquot counted for radioactivity after evaporation.

##### MS activity

SH-SY5Y cells were scraped, pelleted and frozen at -80°C prior to assay for MS activity. Approximately 10<sup>6</sup> cells were resuspended in 1 ml of 100 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose. Cells were disrupted by sonication on ice and the homogenate centrifuged at 4°C. Assays were performed under anaerobic conditions, as described previously.<sup>20</sup> The reaction mixture contained 100 mM potassium phosphate, pH 7.2, 500  $\mu$ M homocysteine, 152  $\mu$ M SAM, 3 mM titanium citrate, 250  $\mu$ M (6R,S)-5-<sup>14</sup>CH<sub>3</sub>-H<sub>2</sub>folate and enzyme in a final volume of 1 ml. The reaction was initiated by the addition of CH<sub>3</sub>-H<sub>2</sub>folate, incubated for 60 min at 37°C and terminated by heating at 98°C for 2 min. Radiolabeled methionine was separated on a Dowex 1-X8 column, which was eluted with 2 ml of water. Control assays, in which sample enzyme was omitted, served as blanks.

##### [<sup>14</sup>C] Formate Autoradiography

SH-SY5Y cells in six-well plates were incubated with Hank's solution containing [<sup>14</sup>C] formate (5  $\mu$ Ci/ml) for 30 min and the reaction terminated by the addition of 1 ml of ice-cold lysis buffer. After scraping, the lysate was centrifuged at 30 000  $\times$  g for 30 min and the pellet resuspended in 1 ml of PBS after which an aliquot was dissolved in sample buffer and separated by SDS-PAGE. A blot containing [<sup>14</sup>C]-labeled membrane proteins was subsequently analyzed by phosphorimaging.

##### Global DNA methylation

As described previously,<sup>21</sup> DNA was extracted from cultured cell pellets using a phenol:chloroform:isoamyl alcohol protocol. DNA (1  $\mu$ g) was enzymatically hydrolyzed by sequential digestion with nuclease P1, venom phosphodiesterase I and alkaline phosphatase, and 20  $\mu$ l of the digest was injected onto a reversed-phase analytical HPLC column (Suplex pKb 100). Isocratic elution was carried out with a mobile phase of ammonium acetate (7 mM; pH=6.7) and methanol (5% v/v) in water. For mass spectrometry, stable isotopes 15N<sub>3</sub> 2'-deoxycytidine and methyl-D<sub>3</sub>, ring-6-D<sub>1</sub> 5-methyl-2'-deoxycytidine were used as internal standards. Ions of *m/z* 126 and 130 were used to detect 5-methyl-2'-deoxycytidine and its isotopomer, and ions of *m/z* 112 and 115 were used to detect 2'-deoxycytidine and its stable isotope, respectively. DNA methylation status was computed as the amount of 5-methylcytidine/ $\mu$ g DNA.

**Methylation-sensitive PCR**

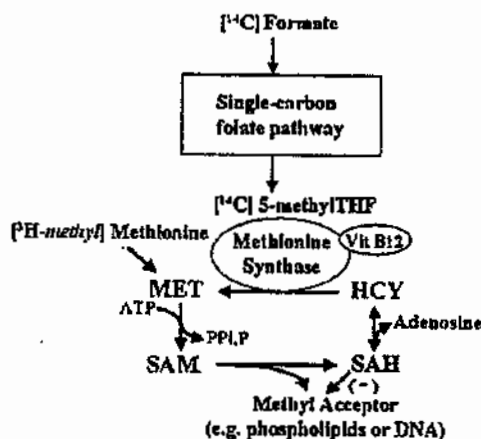
MDA-MB-231, MDA-MB-435 and MCF-7 cells ( $1.5 \times 10^6$ ) were plated onto 10 cm dishes and allowed to attach overnight prior to treatment with wortmannin or LY 294002 for either 16 h or 30 h. Cells were then scraped, divided into two aliquots and pelleted. One pellet was extracted with TRI REAGENT, to isolate RNA (for RT-PCR), and the other was lysed in 500  $\mu$ l TNES (10 mM Tris pH 8, 150 mM NaCl, 2 mM EDTA, 0.5% SDS), for DNA extraction (for methylation-sensitive PCR). RNA samples were reverse transcribed by an initial reaction with dNTPs and oligo-dT at 85°C followed by the addition of DTT, RNase inhibitor and Superscript II at 42°C for 50 min and subsequently 72°C for 15 min to yield cDNA. RT-PCR was performed on the cyclin D2 gene using primers 5'-CATGGAGCTGCTGTGCCACG (sense) and 5'-CCGACCTACCTCCAGCATCC (antisense) and, as a control, the 36B4 primers 5'-GATTGGCTACC-CAACTGTTGCA (sense) and 5'-CAGGGCCAGCAGC-CACAAAGGC (antisense) as described previously.<sup>22</sup> For methylation-sensitive PCR, samples in TNES were incubated with proteinase K (20  $\mu$ l of 20 mg/ml) overnight at 55°C prior to DNA extraction and resuspension in TE buffer. DNA samples were then treated with sodium bisulfite and subsequently extracted using a Wizard DNA cleanup kit, followed by ethanol precipitation and resuspension in ddH<sub>2</sub>O. Methylation-sensitive PCR studies were then performed on cyclin D2 using primers specific for methylated and unmethylated DNA. Products were resolved on 2% agarose gels and visualized by ethidium bromide staining.

**Results****IGF-1 stimulates MS**

MS utilizes 5-methyltetrahydrofolate as a required cofactor, so it is possible to assess its activity in intact cells by labeling the single-carbon folate pool with [<sup>14</sup>C]formate and measuring the subsequent appearance of label in methylated products (Figure 1). Using this strategy, we evaluated the effect of IGF-1 on folate-dependent PLM in SH-SY5Y human neuroblastoma cells. Exposure to IGF-1 produced a dose-dependent increase in folate-dependent PLM with an EC<sub>50</sub> = 0.4 nM, but insulin and IGF-2 did not share this activity (Figure 2a). However, when PLM was measured using [<sup>3</sup>H-methyl]methionine, which bypasses MS (Figure 1), IGF-1 had no effect, although cycloleucine, an inhibitor of methionine adenosylation, reduced methylation (Figure 2b). This specificity of IGF-1 for the stimulation of folate-dependent PLM suggests an action at the level of MS. The tyrosine kinase inhibitor genistein blocked IGF-1 stimulation of folate-dependent PLM (Figure 2c), consistent with an essential role for receptor autophosphorylation.

IGF-1 activates both PI3-kinase and MAP-kinase signaling in SH-SY5Y cells.<sup>23</sup> The selective PI3-kinase inhibitors wortmannin and LY294002 caused dose-

IGF-1 and dopamine regulate methionine synthase  
M. Waly et al.



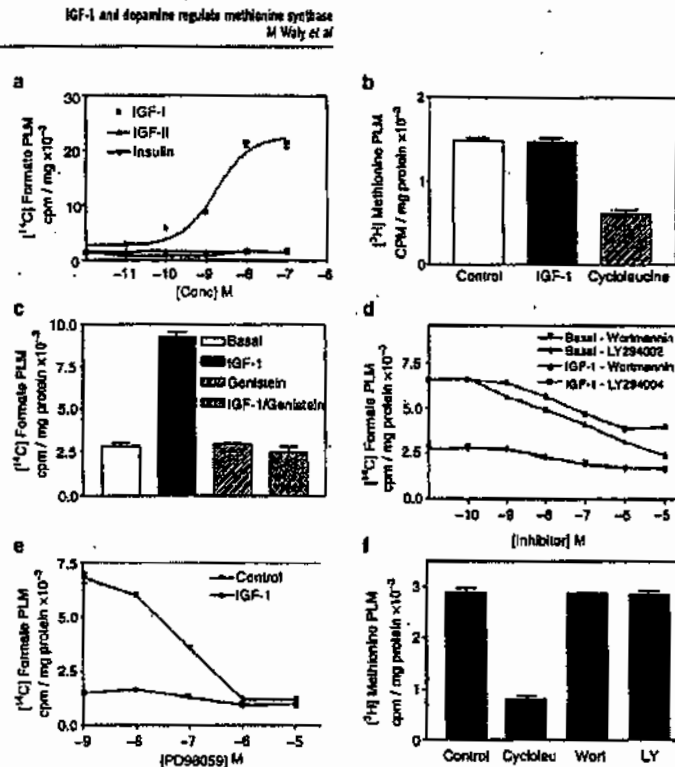
**Figure 1** Folate-dependent and folate-independent PLM. SAM provides methyl groups to numerous acceptors, including phospholipids and DNA. Its methyl group originates from either the folate pathway in the form of 5-methyltetrahydrofolate (5-methylTHF), via MS-dependent methylation of homocysteine (HCY) or from methionine (MET). SAM is converted to SAH, an inhibitor of methyl transfer reactions, which is reversibly hydrolyzed to homocysteine and adenosine. By decreasing homocysteine levels, MS can promote methylation.

dependent decreases in the basal level of MS-dependent PLM and blocked the IGF-1-induced increase (Figure 2d). PD98059, a specific inhibitor of MEK (MAP-kinase kinase), also inhibited the IGF-1-induced increase of PLM (Figure 2e). In contrast, inhibition of PI3-kinase or MAP-kinase pathways did not affect folate-independent PLM, measured with [<sup>3</sup>H-methyl]methionine (Figure 2f). Thus, both PI3-kinase and MAP-kinase activities are required for the IGF-1 stimulation of folate-dependent PLM.

To assess the influence of IGF-1 on MS, SH-SY5Y cells were treated identically to PLM studies and assayed for enzyme activity, measured as the conversion of homocysteine to methionine. As shown in Table 1, IGF-1 increased MS activity to 212% of the basal level. Remarkably, wortmannin and PD98059 each not only blocked the IGF-1-induced increase but also eliminated basal enzyme activity. These effects are in close accord with PLM results and indicate a critical role for PI3-kinase and MAP-kinase signaling pathways in regulating MS.

**Dopamine stimulates MS**

Dopamine caused a dose-dependent increase of folate-dependent PLM in SH-SY5Y cells with an EC<sub>50</sub> of 0.8  $\mu$ M (Figure 3a). Similar to IGF-1, dopamine-stimulated PLM was dependent on both PI3-kinase and MAP-kinase, as indicated by the inhibitory effects of wortmannin, LY294002 and PD98059 (Figures 3b and c).



**Figure 2** IGF-1 stimulates folate-dependent PLM. (a) Folate-dependent PLM, measured with [<sup>14</sup>C]formate, ± IGF-1, IGF-2 or insulin. (b) Folate-independent PLM, measured with [<sup>3</sup>H-methyl]methionine, ± IGF-1 (10 nM) or the methionine adenosyltransferase inhibitor cycloleucine (2 mM). (c) Folate-dependent PLM ± IGF-1 (10 nM) or genistein (10 μM). (d) Folate-dependent PLM ± PI3-kinase inhibitors wortmannin and LY294002. (e) Folate-dependent PLM ± the MEK inhibitor PD98059. (f) Folate-independent PLM ± wortmannin (1 μM) or LY294002 (1 μM).

To confirm direct D4 receptor involvement in folate-dependent PLM, cells were labeled with [<sup>14</sup>C]formate for 30 min and cell membrane proteins were separated via SDS-PAGE and then transferred to a blot for autoradiography. As shown in Figure 3d, only a single 41 kDa protein, corresponding to the D4 receptor, was radiolabeled under these conditions. Dopamine increased receptor labeling, while labeling was reduced by clozapine, a D4 receptor antagonist, and by cycloleucine, an inhibitor of methionine adenosyltransferase. IGF-1 increased D4 receptor-associated radiolabel, consistent with its activation of MS, while wortmannin and PD98059 blocked both dopamine- and IGF-1-stimulated labeling (Figure 3e). Dopamine increased MS activity 2.5-fold and the increase was blocked by wortmannin (Table 1). When added in combination, dopamine and IGF-1 increased MS activity 4.5-fold, indicative of separate but additive mechanisms of PI3-kinase activation. Thus IGF-1 and dopamine both regulate MS activity, and IGF-1 promotes folate-dependent methylation of the D4 dopamine receptor.

#### Effects of ethanol

Ethanol inhibits PI3-kinase-dependent IGF-1 signaling in SH-SY5Y cells,<sup>22</sup> and we evaluated its effect on basal, IGF-1- and dopamine-stimulated, folate-dependent PLM. Ethanol potently reduced folate-dependent PLM activity, and at the highest concentration tested (0.5% v/v), basal- and IGF-1-stimulated PLM were reduced by 67 and 65%, respectively (Figure 4a). The IC<sub>50</sub> for the inhibition of folate-dependent PLM (0.04% or 8.8 mM) reflects one of the most highly ethanol-sensitive responses reported to date. Conversely, ethanol had no effect on folate-independent PLM, measured with [<sup>3</sup>H-methyl]methionine, at concentrations up to 0.5% (Figure 4b). When combined with wortmannin or LY294002, ethanol produced no additional decrease in PLM (Figure 4c). In MS assays, a 60 min exposure to 0.1% ethanol reduced the activity to an undetectable level and there was no response to IGF-1 (Table 1). Thus, ethanol potently interferes with the ability of the IGF-1/PI3-kinase system to augment MS activity directed toward homocysteine.

Table 1 MS activity in SH-SY5Y human neuroblastoma cells

Treatment	MS activity <sup>a</sup> (pmol/min/mg)
Basal	29.1 ± 2.7 (100%)
IGF-1 (10 nM; 30 min)	61.9 ± 2.6 (212%)
Wortmannin (0.1 μM; 60 min)	ND
IGF-1 + wortmannin	ND
Dopamine (10 μM; 30 min)	74.1 ± 2.6 (254%)
Dopamine + wortmannin	ND
Dopamine + IGF-1	132.1 ± 7.7 (454%)
PD98059 (1 μM; 60 min)	ND
IGF-1 + PD98059	ND
Dopamine + PD98059	ND
Ethanol (0.1%; 60 min)	ND
IGF-1 + ethanol	ND
Dopamine + ethanol	ND
CuCl <sub>2</sub> (10 μM; 60 min)	37.0 ± 3.4 (127%)
IGF-1 + CuCl <sub>2</sub>	56.8 ± 4.7 (194%)
Dopamine + CuCl <sub>2</sub>	71.2 ± 5.6 (244%)
CuCl (10 μM; 60 min)	1.9 ± 1.6 (7%)
IGF-1 + CuCl	33.1 ± 3.5 (114%)
Dopamine + CuCl	32.3 ± 3.3 (111%)
HgCl <sub>2</sub> (10 μM; 60 min)	ND
IGF-1 + HgCl <sub>2</sub>	ND
Dopamine + HgCl <sub>2</sub>	ND
PbNO <sub>2</sub> (10 μM; 60 min)	2.7 ± 0.1 (9%)
IGF-1 + PbNO <sub>2</sub>	26.4 ± 0.1 (90%)
Dopamine + PbNO <sub>2</sub>	35.4 ± 2.5 (122%)
Thimerosal (10 nM; 60 min)	ND
IGF-1 + thimerosal	ND
Dopamine + thimerosal	ND

<sup>a</sup>Results are the mean ± sem of replicate measurements from two to four separate experiments. ND=no detectable enzyme activity.

In contrast to its inhibition of basal- and IGF-1-stimulated, folate-dependent PLM, ethanol did not reduce dopamine-stimulated PLM, but instead caused a modest increase (Figure 4d). Since dopamine-stimulated PLM involves the methylation of the D4 receptor, not homocysteine, this implies that ethanol impairs the methylation of homocysteine, but not the

methylation of the D4 receptor. Indeed, after exposure to 0.1% ethanol, dopamine no longer stimulated homocysteine methylation (Table 1). Ethanol there-

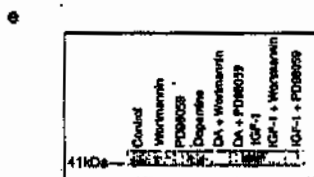
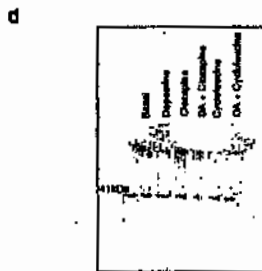
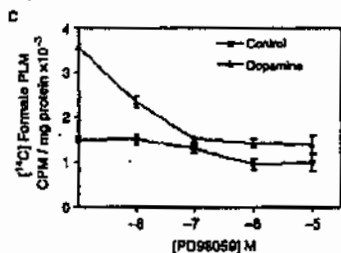
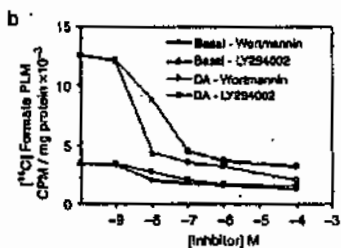
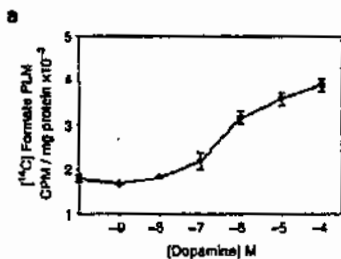


Figure 3 D4 dopamine receptor-mediated, folate-dependent PLM. (a) Dopamine-stimulated PLM measured with [<sup>14</sup>C]formate. (b) Folate-dependent PLM ± dopamine (10 μM) ± wortmannin and LY294002. (c) Folate-dependent PLM ± dopamine (10 μM) ± PD98059. (d, e) Autoradiograms of SH-SY5Y membrane proteins after a 30 min incubation with [<sup>14</sup>C]formate. (d) Dopamine (DA) (10 μM), clozapine (1 μM), cycloleucine (2 mM). (e) Wortmannin (1 μM), PD98059 (1 μM), dopamine (10 μM) and IGF-1 (10 nM).



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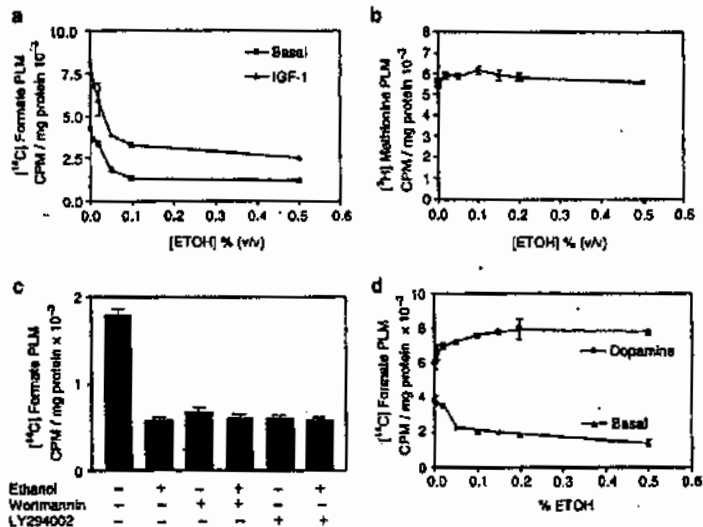


Figure 4 Effects of ethanol on PLM. (a) Folate-dependent PLM ± IGF-1 (10 nM) ± ethanol (ETOH). (b) Folate-independent PLM ± ethanol (0.1%), wortmannin (1 μM), LY294002 (1 μM). (c) Folate-dependent PLM ± ethanol (0.1%), wortmannin (1 μM), LY294002 (1 μM). (d) Folate-dependent PLM ± dopamine (10 μM) ± ethanol.

fore diverts folate-derived methyl groups toward D4R-mediated PLM, away from homocysteine methylation.

#### Effects of metal ions and thimerosal

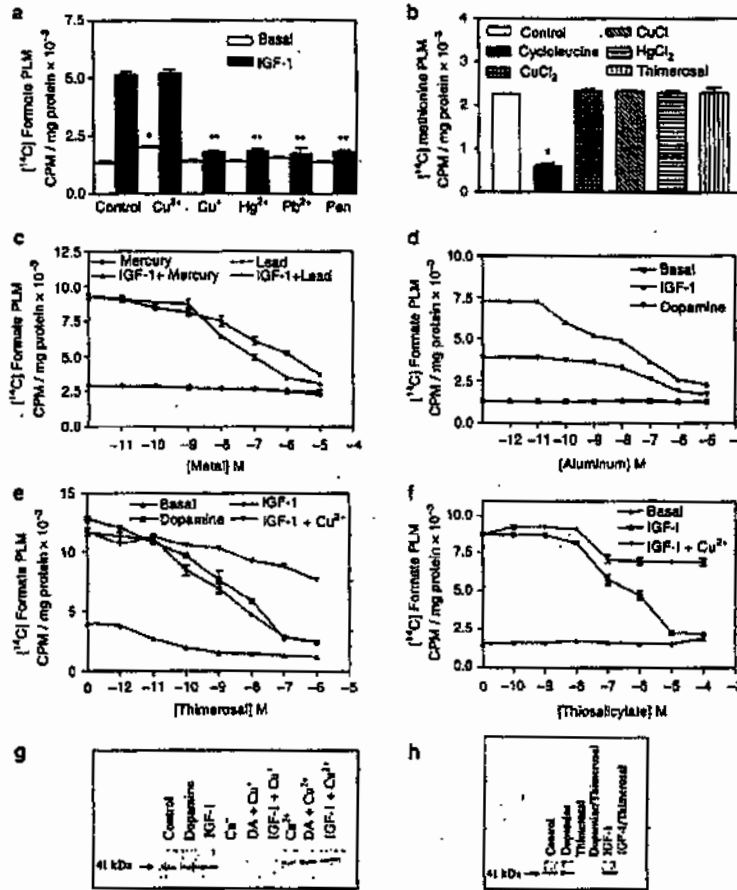
Heavy metal exposure during early development is associated with impaired neurological and cognitive function,<sup>22-24</sup> and Cu<sup>2+</sup> has been previously shown to increase PI3-kinase activity.<sup>25</sup> To evaluate a possible effect of metal ions on MS-dependent PLM, SH-SY5Y cells were incubated with Cu<sup>2+</sup>, Cu<sup>+</sup>, Hg<sup>2+</sup> and Pb<sup>2+</sup> at a concentration of 10 μM for 30 min prior to and during [<sup>14</sup>C]formate labeling in the presence or absence of IGF-1. As shown in Figure 5a, Cu<sup>2+</sup> increased basal PLM while other metal ions had no effect. All metals, with the exception of Cu<sup>2+</sup>, inhibited the stimulatory action of IGF-1. Pretreatment with penicillamine, which binds Cu<sup>2+</sup>, completely eliminated the IGF-1 response. None of the metal ions affected PLM measured with [<sup>3</sup>H-methyl]-methionine (Figure 5b), indicating their specificity for MS-related methylation events. Cu<sup>+</sup> blocked radiolabeling of the D4 receptor, while Cu<sup>2+</sup> was without effect (Figure 5g).

In dose-response studies, Hg<sup>2+</sup> and Pb<sup>2+</sup> potently inhibited IGF-1-stimulated, folate-dependent PLM with IC<sub>50</sub> values of 15 and 100 nM, respectively (Figure 5c). Aluminum inhibited IGF-1-stimulated PLM in a biphasic manner, with IC<sub>50</sub> values of 0.1 and 200 nM (Figure 5d). Against dopamine-stimulated PLM, however, Al<sup>3+</sup> exhibited monophasic inhibition with an IC<sub>50</sub> of 150 nM.

It has been suggested that increases in the incidence of ADHD and autism might be linked to the ethylmercury-containing preservative thimerosal,<sup>7,26,27</sup> a component of vaccines formulated in multidose containers. Thimerosal potently inhibited basal, IGF-1- and dopamine-stimulated, folate-dependent PLM, with a threshold of approximately 10 pM and an IC<sub>50</sub> of 1 nM (Figure 5e), and also blocked folate-dependent radiolabeling of the D4R (Figure 5h). Similar to metal ions, thimerosal had no effect on folate-independent PLM (Figure 5b). When Cu<sup>2+</sup> (1 μM) was added, the extent of thimerosal inhibition was reduced (Figure 5e), suggesting that heavy metals compete with Cu<sup>2+</sup> in the PI3-kinase pathway leading to MS activation.

Thimerosal is composed of ethylmercury bound to thiosalicylate, a metal chelator that is similar in structure to penicillamine. Thiosalicylate inhibited IGF-1-stimulated, folate-dependent PLM, albeit with 500- to 100-fold lower potency than thimerosal, but did not affect basal PLM (Figure 5f). This effect of thiosalicylate was greatly reduced in the presence of 1 μM Cu<sup>2+</sup>, suggesting that the chelation of Cu<sup>2+</sup> may underlie its inhibitory effect.

Cu<sup>2+</sup> modestly increased MS activity and did not interfere with stimulation by IGF-1 and dopamine (Table 1). Cu<sup>+</sup> and Pb<sup>2+</sup> reduced the basal activity by more than 90%, but allowed nearly normal increases by IGF-1 and dopamine. Thimerosal and Hg<sup>2+</sup> each reduced MS activity to an undetectable level and completely blocked stimulatory effects of IGF-1 and dopamine (Table 1). Based on these results, the



**Figure 5** Effects of heavy metal ions and thimerosal on PLM. (a) Folate-dependent PLM ± IGF-1 (10 nM) in the presence of CuCl<sub>2</sub>, CuCl, HgCl<sub>2</sub> or PbCl<sub>2</sub> (10 μM), or penicillamine (1 mM). \*Significant increase from control ( $P < 0.05$ ); \*\*Significant decrease from IGF-1 only ( $P < 0.01$ ). (b) Folate-independent PLM ± cycloleucine (2 mM), CuCl<sub>2</sub>, CuCl, HgCl<sub>2</sub>, thimerosal (10 μM). \*Significant decrease from control ( $P < 0.01$ ). (c-f) Folate-dependent PLM in the presence of IGF-1 (10 nM) or dopamine (10 μM) ± mercury, lead, aluminum, Cu<sup>2+</sup> (1 μM), thimerosal or thiosalicylate. (g) Radiolabeling of the D4 dopamine receptor in the presence of dopamine (10 μM), IGF-1 (10 nM), CuCl (1 μM), CuCl<sub>2</sub> (1 μM). (h) Dopamine (10 μM), thimerosal (10 nM) and IGF-1 (10 nM).

Inhibitory effects of metal ions and thimerosal on folate-dependent PLM can be attributed to the inhibition of MS activity.

**DNA methylation**

Since increased MS activity can lower the levels of SAH, an inhibitor of methylation reactions, we examined the influence of IGF-1 and dopamine on global DNA methylation status. After a 6 h exposure, IGF-1 increased global DNA methylation by 107%, while dopamine caused an increase of 41% (Table 2). Wortmannin caused a modest increase in DNA methylation, and blocked IGF-1- and dopamine-

induced increases. Ethanol had no effect on its own but, similar to wortmannin, blocked the ability of IGF-1 to increase DNA methylation. In contrast, ethanol did not block the stimulatory effect of dopamine. Thus the ability of both IGF-1 and dopamine to increase MS activity is associated with substantial increases in DNA methylation, suggesting that PI3-kinase signaling may alter gene expression via this mechanism. Moreover, changes in DNA methylation parallel the effects of these agents on folate-dependent PLM.

To evaluate the ability of PI3 kinase to affect DNA methylation and gene expression, we used methyla-

8

Table 2 Global DNA methylation in SH-SY5Y cells

Drug treatment	Global DNA methylation <sup>a</sup> (ng MeCyt/ $\mu$ g DNA)
Control	1.38 (100%)
IGF-1 (0.1 nM)	2.87 (207%)
Wortmannin (0.1 $\mu$ M)	1.69 (123%)
IGF-1 + wortmannin	1.23 (89%)
Dopamine (10 $\mu$ M)	1.94 (140%)
Dopamine + wortmannin	1.39 (101%)
Ethanol (0.1% v/v)	1.40 (102%)
IGF-1 + ethanol	0.91 (66%)
Dopamine + ethanol	2.78 (201%)

<sup>a</sup>Each data point is the mean of replicate determinations from duplicate samples.

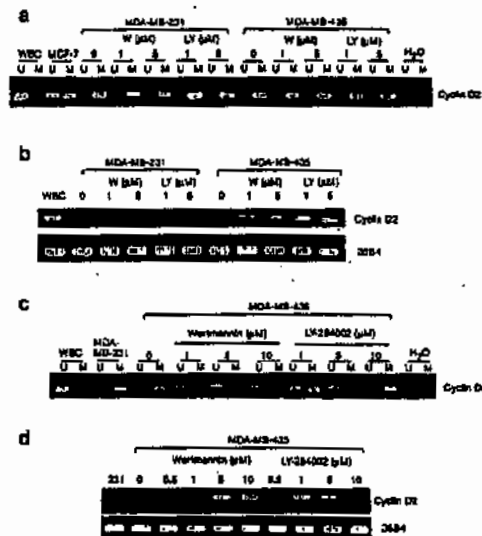


Figure 8 Methylation-specific PCR (MSP) and RT-PCR analysis of cyclin D2 following wortmannin and LY 294002 treatment. Cells (MDA-MB-231 and MDA-MB-435) were treated with 0–5  $\mu$ M wortmannin (W) or LY 294002 (LY) for 16 h. The MSP analysis of cyclin D2 was performed using primers specific for unmethylated (U) or methylated (M) DNA amplification (a). RT-PCR was performed to detect cyclin D2 expression and, as a control, the housekeeping gene 36B4 (b). MDA-MB-435 cells were treated with 0–10  $\mu$ M of Wortmannin and LY-294002 for the longer time period of 30 h. MSP analysis (c) and RT-PCR (d) was then performed as above on the cyclin D2 gene. WBC: white blood cell control that is unmethylated and expresses cyclin D2. Untreated MCF-7 cells are hemimethylated for cyclin D2 (a).

tion-sensitive PCR to determine the methylation status of the cyclin D2 gene, which contains a methylation-sensitive promoter.<sup>22,23</sup> As illustrated in Figure 8a, a 16 h treatment with LY294002, but not wortmannin, increased the proportion of demethylated cyclin D2 promoter in the breast cancer-derived MDA-MB-231 and MDA-MB-435 cell lines. RT-PCR showed that both wortmannin and LY294002 increased gene expression in MDA-MB-435 cells (Figure 8b). After a 30 h incubation, wortmannin and LY294002 both caused demethylation of the cyclin D2 promoter in MDA-MB-435 cells, along with an increase in transcription (Figures 8c and d). This confirms the ability of PI3 kinase to regulate DNA methylation and gene expression in a non-neural cell line.

Discussion

MS links the single-carbon folate pathway to the methionine cycle, and is a potentially important site for metabolic control. Nonetheless, there have been no prior reports of its regulation by extracellular signaling pathways. Our studies demonstrate the ability of IGF-1 and dopamine to increase MS activity via a mechanism that requires the activity of both PI3-kinase and MAP-kinase pathways. MS activity is a major determinant of both homocysteine and SAH levels, and the efficiency of methylation reactions is governed by the [SAM] to [SAH] ratio.<sup>12,73</sup> These relationships imply that growth factors, by increasing PI3- and MAP-kinase activity, can facilitate transmethylation reactions, via activation of MS. Conversely, agents interfering with this mechanism will impair methylation.

Our studies also provide evidence that ethanol, heavy metals and the vaccine preservative thimerosal potently interfere with MS activation and impair folate-dependent methylation. Since each of these agents has been linked to developmental disorders, our findings suggest that impaired methylation, particularly impaired DNA methylation in response to growth factors, may be an important molecular mechanism leading to developmental disorders.

DNA methylation is a crucial regulator of gene expression that has been linked to several developmental disorders. The majority of Rett syndrome cases are caused by *MeCP2* mutations that interfere with MeCP2 to binding to methylated CpG sites in the genome.<sup>7</sup> As a consequence, the protein complex necessary for histone modification and gene silencing fails to form, leading to dysregulated gene expression. Fragile-X syndrome is associated with localized hypermethylation of unstable CCG repeats at fragile sites on the X-chromosome (Xq27.3).<sup>4</sup> Impaired MS activity could therefore contribute to developmental disorders via altered patterns of DNA methylation.

Growth factors (eg nerve growth factor, brain-derived neurotrophic factor and IGF-1) promote development of the neuronal phenotype and support the function and survival of differentiated nerves.<sup>34–38</sup>

The capacity to activate simultaneously both PI3-kinase and MAP-kinase pathways is a feature of many growth factors.<sup>27-30</sup> Blocking the methionine cycle (eg with inhibitors of SAH hydrolase) interferes with neurotrophic responses,<sup>19,71</sup> indicating an essential role for methylation in growth factor action. Since differences in cellular phenotype reflect varied patterns of methylation-dependent gene silencing, it is reasonable to hypothesize that growth factors might directly or indirectly modulate genomic methylation status during development.

IGF-1 exerts trophic and antiapoptotic effects on a wide variety of cell types, and its involvement in brain development is well documented.<sup>40,41</sup> In addition to its neurotrophic action, IGF-1 promotes differentiation and survival of myelin-producing oligodendrocytes,<sup>42</sup> an action in which divalent copper plays an integral role. Thus the chelation of copper causes demyelination and an upregulation of IGF-1.<sup>43</sup> Vitamin B12 deficiency<sup>44</sup> and chronic nitrous oxide exposure,<sup>45</sup> both of which impair MS, also cause demyelination. We found that  $\text{Cu}^{2+}$  promotes MS activity (Table 1) and protects against the inhibitory effects of other metals (Figure 5e), while  $\text{Cu}^{2+}$  chelation has an opposite effect (Figures 5a and f). Thus oligodendrocytes provide a specific example of how IGF-1, metal ions and methylation can combine to affect cellular differentiation and brain development.

During postnatal development, myelination is critical for the specification of fixed connections between brain regions (ie hard-wiring), and there have been a number of reports of abnormal white matter (ie myelination) in autism.<sup>46-48</sup> Neurodevelopmental insults affecting myelination could lead to abnormal neural connections, resulting in the enhancement of certain relationships, but deficiencies in others, as is frequently observed in autism. Reduced IGF-1 levels have been reported in autism,<sup>49</sup> which may also contribute to impaired myelination.

Fetal ethanol exposure, consequent to maternal alcohol use, leads to the complex disorder known as fetal alcohol syndrome.<sup>5</sup> In humans and animal models, IGF-1 levels are reduced after fetal ethanol exposure, and the decrease is sustained through postnatal development.<sup>50,51</sup> Ethanol increases homocysteine levels in animals and man,<sup>52,53</sup> in association with impaired MS activity.<sup>54</sup> Ethanol potently inhibits basal- and IGF-1-stimulated MS activity (Table 1), reduces folate-dependent methylation (Figure 3a), and blocks the ability of IGF-1 to increase DNA methylation (Table 2). The  $\text{IC}_{50}$  for ethanol inhibition of methylation (8 mM) corresponds to blood levels produced by only one or two drinks, indicating a potential for adverse effects on methylation events from only moderate drinking. In a related finding, IGF-1 has been shown to promote recovery from carbon tetrachloride-induced cirrhosis, by increasing DNA methylation and normalizing gene expression.<sup>55</sup>

As illustrated in Figure 7, MS has two substrates, homocysteine and the dopamine D4 receptor in its

IGF-1 and dopamine regulate methionine synthase  
M Waly et al



9

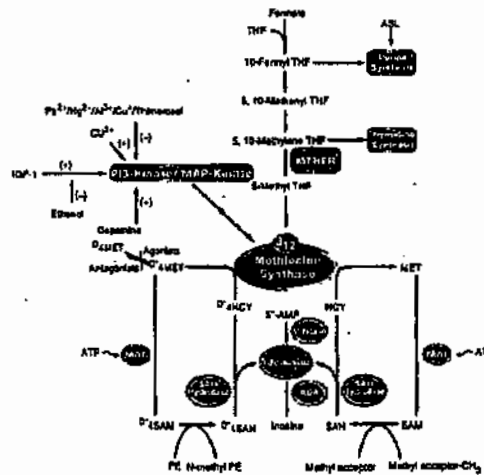


Figure 7 IGF-1 and dopamine regulate MS. Formate provides carbon atoms to the folate pathway that are used for purine and thymidine synthesis or are irreversibly reduced by 5,10-methylenetetrahydrofolate reductase (MTHFR) to 5-methyl-THF. MS utilizes 5-methyl-THF for the methylation of homocysteine (Hcy; right) and the D4 dopamine receptor during dopamine-stimulated PLM (D4R; left). D4R-mediated PLM requires the active receptor conformation (indicated by \*) and is promoted or inhibited by receptor agonists or antagonists, respectively. IGF-1 and dopamine augment MS activity via a PI3-kinase and MAP kinase-dependent mechanism, increasing methionine synthesis and lowering SAH levels.  $\text{Cu}^{2+}$  promotes MS activity, while  $\text{Pb}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Al}^{3+}$ ,  $\text{Cu}^{-}$  and thimerosal reduce activity. ATP-dependent adenosylation of methionine by methionine adenosyltransferase (MAT) forms SAM, the universal methyl donor for many reactions, including DNA methylation. SAH hydrolase reversibly catalyzes adenosine removal from SAH. Abnormalities involving purine synthesis (eg ASL mutations) or adenosine metabolism (increased 5'-nucleotidase (5'-Ntase) or decreased adenosine deaminase (ADA) activity) can adversely affect the capacity for methylation and thereby synergize with reduced MS activity.

homocysteine state. Dopamine-stimulated PLM, measured with [ $^{14}\text{C}$ ]formate, reflects D4 receptor-directed MS activity and ethanol increases dopamine-stimulated PLM (Figure 4d), in contrast to its inhibition of homocysteine-directed MS activity (Table 1). These results indicate that ethanol promotes the ability of MS to utilize the D4 receptor as a substrate, while simultaneously decreasing homocysteine methylation.

Dopamine increases MS activity and folate-dependent PLM in SH-SY5Y cells via a mechanism requiring both PI3-kinase and MAP-kinase activation (Table 1, Figures 3b and c), and this increase will promote the efficiency of the D4 receptor-mediated PLM cycle. Although the functional role of dopamine-

stimulated PLM is not fully understood, the incidence of ADHD is linked to genetic variations within the D4 receptor gene,<sup>18</sup> and it has been proposed that dopamine-stimulated PLM plays a central role in attention.<sup>19</sup> Impairment of MS could therefore adversely affect the capacity for attention and could contribute to the risk of ADHD.

Lead exposure, particularly early in life, causes growth retardation along with impairments in attention and cognitive development,<sup>5</sup> and government guidelines establish blood concentrations exceeding 500 nM as indicative of lead poisoning.<sup>20</sup> An increase in blood lead levels from 1 to 10 µg/dl (120–1200 nM) is associated with an IQ decrease of 7.4 points.<sup>20</sup> Since lead inhibits IGF-1-stimulated methylation with an IC<sub>50</sub> value of 100 nM (Figure 5c), impaired MS could contribute to developmental delay and impaired cognition associated with lead poisoning.

Mercury exposure has been suggested as a possible cause of autism<sup>7</sup> and methylmercury is a well-recognized neurotoxin.<sup>21</sup> A blood mercury level of 28 nM has been recommended by the Environmental Protection Agency as a reference value for defining toxic exposure.<sup>22</sup> We found that inorganic mercury inhibits IGF-1-stimulated methylation with an IC<sub>50</sub> of 15 nM (Figure 5c).

Aluminum salts are used as vaccine adjuvants, based on their ability to improve dendritic cell response to presented antigens. The aluminum content of vaccines varies from 0.125 to 0.85 mg/dose, which would produce concentrations of approximately 0.7 to 4.5 µM, if uniformly distributed in the body water of a 7 kg infant. These concentrations produce greater than 50% inhibition of both IGF-1- and dopamine-stimulated methylation, raising the possibility that aluminum concentrations produced by vaccination might adversely affect methylation events. In light of the importance of MS in regulating DNA methylation<sup>23,24</sup> and the central role of DNA methylation in development,<sup>9</sup> we propose that metal exposures, including lead, mercury and aluminum, may contribute to developmental syndromes via their inhibitory effects on signaling pathways that regulate MS activity.

Thimerosal is an ethylmercury derivative of thio-salicylate, widely used as a preservative to block the growth of contaminating organisms in biological products. It was included in most vaccines in the US until 1999, when the FDA initiated a precautionary program calling for 'thimerosal-free' vaccines. Most, but not all, vaccines are now 'thimerosal-free', meaning that they contain less than 0.5 µg thimerosal/dose.<sup>25</sup> Thimerosal inhibits IGF-1 and dopamine-stimulated methylation with an IC<sub>50</sub> near 1 nM, (Figures 5e and f), indicating its potential for disrupting normal growth factor control over methylation. Thio-salicylate itself also inhibited methylation, presumably by chelating Cu<sup>2+</sup>, but was more than 100-fold less potent than thimerosal (Figure 5f), indicating that the ethylmercury in thimerosal is responsible for its inhibitory effect. The presence of

added Cu<sup>2+</sup>, however, significantly offsets thimerosal-induced inhibition, reflecting competition between promotional and inhibitory effects of metals on the PI3-kinase MS pathway. Thus, the toxicity of thimerosal in the body may depend upon the concentrations of metal ions that provide either additive toxicity or protective effects on PI3-kinase signaling. Thimerosal has been reported to activate apoptosis in lymphocytes<sup>26</sup> and in cultured human cortical neurons,<sup>27</sup> consistent with the inhibition of the PI3-kinase signaling pathway.

A single thimerosal-containing vaccination produces acute ethylmercury blood levels of 10–30 nM,<sup>28</sup> and blood samples in 2-month-old infants, obtained 3–20 days after vaccination, contain 3.8–20.6 nM ethylmercury.<sup>29</sup> Our studies therefore indicate the potential for thimerosal to cause adverse effects on MS activity at concentrations well below the levels produced by individual thimerosal-containing vaccines.

If impaired MS activity does indeed contribute to neurodevelopmental toxicity, limitations in other pathways that support homocysteine methylation could predispose individuals to higher risk. Since SAH hydrolase is reversible, the concentration of adenosine determines the probability that homocysteine will be reconverted to SAH (Figure 7). Adenosine deaminase activity is reduced in autism,<sup>30</sup> which would lead to higher adenosine levels and enhanced SAH formation. A polymorphism in the adenosine deaminase gene, that gives rise to a lower activity enzyme, is over-represented in autism.<sup>31,32</sup> Adenosine is formed by the action of 5'-nucleotidase on AMP, and Page *et al.*<sup>33</sup> found eight- to 10-fold higher 5'-nucleotidase activity in association with an 'autism-like' developmental disorder. Each of these autism-associated metabolic abnormalities could synergize with reduced MS activity to impair methylation.

Mutations in the adenylosuccinate lyase (ASL) gene are a rare but penetrant cause of autism.<sup>3</sup> Lower enzyme activity blocks *de novo* purine synthesis in conjunction with a massive buildup of preblock intermediates that are ultimately excreted in the urine. As illustrated in Figure 7, increased flux of folate-derived single-carbon groups to purine synthesis restricts the availability of 5-methylTHF for MS. Moreover, increased flux of single-carbon groups toward *de novo* purine synthesis is common in autism,<sup>34</sup> as well as in Lesch-Nyhan Syndrome,<sup>35</sup> and this may increase sensitivity to neurodevelopmental toxins acting on MS.

A recent rise in autism incidence<sup>36</sup> has triggered concerns that an environmental factor might be promoting developmental disorders. Attention has been directed towards vaccines as a possible cause of the rise, since there has been a significant increase in the number of required vaccinations since the early 1980s.<sup>7,24,25</sup> Depending on infant weight and vaccination schedule, the vaccine-associated dosage of ethylmercury during the initial 24 months of life approached or exceeded federal guidelines for

methylmercury exposure.<sup>61</sup> A recent analysis of data from the Vaccine Adverse Event Reporting System, maintained by the Centers for Disease Control, found a significant correlation between the use of the thimerosal-containing formulation (vs the thimerosal-free formulation) of the Diphtheria, Tetanus and acellular Pertussis (DTaP) vaccine and autism.<sup>31</sup> The discovery of the PI3-kinase/MAP-kinase/MS pathway, and its potent inhibition by developmental neurotoxins, including vaccine components thimerosal and aluminum, provides a potential molecular explanation for how increased use of vaccines could promote an increase in the incidence of autism. The increased incidence of ADHD, which preceded the more recent rise in autism, could represent an alternative manifestation of vaccine-associated neurodevelopmental toxicity, since the D4 dopamine receptor is linked to ADHD<sup>18</sup> and its PLM function depends on MS.<sup>15</sup>

There are important limitations to our findings. We utilized a transformed cell line, and molecular events in tumor-derived cells might not mirror those in normal cells. SH-SY5Y cells are undifferentiated neuronal precursor cells, so it remains unclear whether growth factors and/or dopamine modulate MS activity and DNA methylation in fully differentiated cells. On the other hand, undifferentiated cells may provide a particularly appropriate model system for the study of developmental disorders. It is obvious that biochemical studies under cultured cells conditions do not replicate the complex *in vivo* environment, in terms of ambient metal ion concentrations, redox conditions and other factors that could influence methylation events. Further investigation of the *in vivo* and *in vitro* effects of heavy metals on growth factor-induced cellular differentiation is needed. While our studies focused exclusively on MS- and methylation-related events, we can speculate that other PI3-kinase signaling pathways may also be affected by metal ions.

In summary, IGF-1 and dopamine activate methionine kinase in SH-SY5Y human neuroblastoma cells via a PI3-kinase and MAP-kinase-dependent mechanism, and the activation is associated with increased DNA methylation. Several neurodevelopmental toxins inhibit this newly recognized pathway with remarkable potency, suggesting that their pathological effects might result from interruption of growth factor-initiated increases in DNA methylation and normal epigenetic regulation of gene expression. Further studies are needed to establish the functional significance of regulated MS activity and to evaluate the possibility that vaccine components (ie thimerosal and aluminum) may have contributed to the risk of autism, ADHD and other developmental disorders.

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IGF-I and dopamine regulate methionine synthesis  
N Waly et al



13

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