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Are there altered antibody responses to measles, mumps, or rubella viruses in autism?

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The role that virus infections play in autism is not known. Others have reported that antibodies against measles virus are higher in the sera/plasma of children with autism versus controls. The authors investigated antibody titers to measles, mumps, and rubella viruses and diphtheria toxoid in children with autism, both classic onset (33) and regressive onset (26) forms, controls (25, healthy age- and gender-matched) and individuals with Tourette's syndrome (24) via enzyme-linked immunosorbent assays. No significant differences in antibody titers to measles, mumps, and rubella viruses and diphtheria toxoid were found among the four groups. Additionally, there were no significant differences between the four groups for total immunoglobulin (Ig)G or IgM. Interestingly, the authors did find a significant number (15/59) of autism subjects (classic and regressive onset combined) who had a very low or no antibody titer against rubella virus, compared to a combine control/Tourette's group (2/49). *Journal of NeuroVirology* (2007) **13,** 252–259.

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Introduction

Autism is a behaviorally defined developmental disorder of the central nervous system (CNS) characterized by unusual social, communicative, and behavioral development. The phenotypic heterogeneity of this disorder is one of its most confounding aspects. In most cases the etiology is unknown; however, a role is played by both genetics and environmental factors (Bailey *et al*, 1995; Coleman and Gillberg, 1985; Nelson, 1991). The current estimate of the prevalence rate of autism is more than 10 per 10,000, and there is a biased gender ratio of 3–4:1, boys to girls (Fombonne, 2003; Yeargin-Allsopp *et al*, 2003). Diagnosis of this disorder has increased over the last few decades throughout various countries (Gillberg and Wing, 1999; Chakrabarti and Fombonne, 2001; Yeargin-Allsopp *et al*, 2003). This increase may be due to a combination of changing diagnostic practices, improved identification and availability of services, or an actual increase in incidence (Fombonne, 2003). Controversial claims that the measles-mumps-rubella (MMR) vaccine is responsible for this increase, by triggering a regressive onset form of the disorder, has led to a decline in MMR vaccination rates accompanied by measles infection outbreaks and concern of further disease outbreaks in the United Kingdom (UK) (Thomas *et al*, 1998; Tookey, 2004; Jansen *et al*, 2003). Potentially, viral components within the vaccines could stimulate antibodies that could cross-react with host tissues, thus inducing autoimmunity. Another possibility is that attenuated measles virus or other virus contained in the vaccine could persist and somehow induce autism.

The controversy surrounding the MMR vaccine and autism was instigated by a report correlating onset of autistic symptoms with MMR vaccination

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in eight children based on parental or physician observations (Wakefield *et al*, 1998). Subsequent studies comparing children with autism with a new form or variant of inflammatory bowel disease, ileocolonic lymphonodular hyperplasia, to controls reported (1) increased detection of measles virus fusion and hemagglutinin genes, via TaqMan reverse transcriptase–polymerase chain reaction (RT-PCR), and nucleocapsid gene, via RT *in situ* PCR, in the gastrointestinal (GI) tracts (measles virus positive: 75/91 affected children, 5/70 controls); and (2) the presence of measles virus genomic RNA, via RT-PCR, in peripheral blood mononuclear cells (PBMCs) of 3/9 children with autism having gut manifestations (Kawashima *et al*, 2000; Uhlmann *et al*, 2002). The sequence of the measles virus genomic RNA detected in the PBMCs was consistent with the Schwarz vaccine strain of measles virus (Kawashima *et al*, 2000). Measles virus was suggested to be an immunological trigger for the characteristic gut pathology found in a subset of children with developmental disorders (Uhlmann *et al*, 2002; Wakefield *et al*, 2005). Additionally, a recent review of measles virus infection and vaccination suggests that a preexisting immune dysfunction could account for the persistence of measles virus at unanticipated sites such as the gut, which in turn leads to the development of autism (Kennedy *et al*, 2004). An alternate interpretation is that a preexisting immune dysfunction could lead to both the persistence of measles virus and the development of autism independently of each other.

Another group has used enzyme-linked immunosorbent assays (ELISA) and immunoblotting to detect antibodies to measles virus in the blood of both children with autism and normal controls (Singh *et al*, 2002; Singh and Jensen, 2003). This group's initial study (Singh *et al*, 1998) showed no significant difference in the antibody titer against measles in sera between children with autism and normal controls. Their subsequent studies (Singh *et al*, 2002; Singh and Jensen, 2003) reported that children with autism had a significantly higher level of serum antibodies against measles-mumps-rubella antigens than normal controls, and specifically, a significantly higher level of serum antibodies against measles virus. An inappropriate or abnormal antibody response to the measles portion of the MMR vaccine has been proposed to be related to autism pathogenesis. We have previously explored issues concerning the controls used and the immunoblotting technique employed in these studies (Sweeten and Fujinami, 2004).

In contrast to the above studies supporting a link between the MMR vaccine and autism, numerous other studies, primarily epidemiological in nature, have failed to find a causal association between MMR immunization and autism (Smeeth *et al*, 2004; Mäkelä *et al*, 2002; Madsen *et al*, 2002; Taylor *et al*, 1999, 2002; Fombonne and Chakrabarti, 2001; Dales *et al*, 2001; Kaye *et al*, 2001; Peltola *et al*, 1998; Peterson and Torrey, 1976; reviewed in Madsen and Vestergaard, 2004). Not only is there a lack of a sudden "step-up" in incidence of autism following the introduction of the MMR vaccine, but developmental regression also does not cluster in the months following MMR vaccination (Taylor *et al*, 1999). MMR vaccination was not found to be related to either bowel problems or autistic regression (Taylor *et al*, 2002; Richler *et al*, 2006). Additionally, although the incidence of autism increased between 1988 and 1993 in a study of boys with autism born in the UK, the prevalence of MMR immunization was constant at over 95% (Kaye *et al*, 2001). This held true for children born in California between 1980 and 1994, as well (Dales *et al*, 2001). Recently, a large retrospective cohort study of 537,303 children born in Denmark between 1991 and 1998 showed no increased risk of autism among MMR-vaccinated children (82% of the children studied) compared to unvaccinated children (Madsen *et al*, 2002). Thus, all epidemiological studies conducted on this issue have found no correlation between vaccination and autism.

To investigate the role of measles in autism further, we measured plasma antibody levels to measles virus and other pathogens, in children with autism, both classic onset and regressive onset forms, compared to control children and children with Tourette's syndrome. Differences between classic and regressive autism were tested to investigate possible immune/autoimmune characteristics of these two groups. Regression was defined as the loss of basic abilities in language, social interaction, and imaginary play, after a period of apparent normal development for at least the first 15 months post partum (Goldberg *et al*, 2003; Luyster *et al*, 2005). There is loss of the previous ability to communicate and an onset of restrictive and repetitive behaviors (Goldberg *et al*, 2003; Luyster *et al*, 2005). Children with classic and regressive autism were also tested against controls and children with Tourette's syndrome.

Tourette's syndrome children serve as a neurological control. Tourette's syndrome is a neurodevelopmental disorder of unknown etiology that has childhood onset and is characterized by multiple motor and vocal tics and behavioral problems (Jankovic, 2001; American Psychiatric Association, 2000). As in autism, Tourette's syndrome has a heterogeneous phenotype, a gender ratio of 3:1, boys to girls, and a strong genetic etiology (Jankovic, 2001). Children with Tourette's syndrome are also interesting based on evidence that autoimmune mechanisms could be involved in the pathogenesis of the disease (Kiessling *et al*, 1993; Swedo *et al*, 1998; Yeh *et al*, 2006).

In addition, we determined total levels of immunoglobulin (Ig)G and IgM present in the plasma. Imbalances in the levels of various Igs may indicate an underlying autoimmune disorder or an altered susceptibility to infections. Trajkovski *et al* (2004) reported that there were significantly higher levels of IgG and IgM in the plasma of children with autism, compared to unaffected siblings.

Table 1 Antibody to measles, mumps, and rubella viruses and diphtheria toxoid

	Classic	Regressive	Control	Tourette's	*F* value	*P* value
Measles[1,4]	708 ± 458 (33)	683 ± 473 (26)	750 ± 407 (24)	846 ± 542 (22)	0.55	.65
Mumps[2,5]	1,114 ± 572 (32)	1,151 ± 667 (25)	1,140 ± 524 (25)	1,099 ± 489 (24)	0.05	.99
Rubella[3,4]	238 ± 135 (25)	190 ± 138 (18)	191 ± 137 (24)	181 ± 120 (23)	0.90	.44
Diphtheria[1,6]	931 ± 679 (33)	1,227 ± 745 (26)	1,103 ± 732 (25)	1,171 ± 508 (24)	1.08	.36

[1]Serum dilution was 1:1024.
[2]Serum dilution was 1:512.
[3]Serum dilution was 1:256.
[4]Mean optical densities × 1000, adjusted for age ± standard deviations (sample number).
[5]Mean optical densities × 1000, adjusted for age and antibiotic status ± standard deviations (sample number).
[6]Mean optical densities × 1000 ± standard deviations (sample number).

We found no significant group differences in antibody titers to any of the pathogens tested and no significant differences between the groups for IgG or IgM. However, there was a significant number of children with autism (classic and regressive combined) that had either no or very low antibody titers against rubella virus compared to the combined control and Tourette's group or the control group alone. This study provides further evidence against the role of measles in the etiology or pathogenesis of autism.

Results

There was a significant difference in age between the pairings of classic onset autism and Tourette's and regressive onset autism and Tourette's, as found by our previous analysis of these same subjects (classic autism, $n=33$; regressive autism, $n=26$; control, $n=25$; Tourette's syndrome, $n=24$) (Kirkman *et al*, 2007, in press). The fact that children with Tourette's are usually diagnosed at a later age than children with autism can account for this difference in age (American Psychiatric Association, 2000). No significant differences in the gender ratio were seen.

Antibody titers against measles, rubella, and mumps viruses and diphtheria toxoid in the plasma of children with autism, both classic and regressive, controls and Tourette's syndrome were determined by ELISA in order to show any altered antibody responses to vaccines or wild-type infections. We ran the SAS general linear model (GLM) procedure, predicting the antibody levels from the covariates: age, gender, and medication status. For measles virus and rubella virus, there was a significant age effect ($F=6.28$, $P<.05$; $F=4.67$, $P<.05$, respectively) on antibody titers. Specifically for measles, antibody titers significantly decreased with increasing age. For mumps virus, there was a significant age and antibiotics effect ($F=5.22$, $P<.01$) on antibody titers. None of the covariates tested had a significant effect on antibody titers to diphtheria toxoid. No significant interaction effects among covariates were found, and there were no significant differences in covariate effects within diagnostic groups. Using the residuals from the regression analysis controlling for the effects of the covariates, the autism groups, both classic and regressive, the Tourette's group and the controls were compared by analysis of variance (ANOVA). There were no significant differences between the four groups for covariate adjusted measles virus, mumps virus, rubella virus, or diphtheria toxoid (Table 1).

Interestingly, several of the autism samples had a very low or no antibody titer against rubella virus. In our ANOVA analysis, eight subjects with classic autism and seven subjects with regressive autism were dropped due to a lack of antibody titer for rubella virus, whereas only one each for control and Tourette's subjects were dropped for the same reason. A scattergram plotting the age versus diagnosis of the subjects with very low or no antibody titer against rubella virus shows that the samples are evenly distributed over the age range of 3 to 12 years for both the classic onset autism group and the regressive onset autism group (Figure 1). If we compare the combined autism group (15 with no antibody titer out of 59 subjects) to the combined control/Tourette's group (2 with no antibody titer out of 49 subjects) using a Fisher's exact test, this difference is significant ($P=.002$). If we compare the combined autism group to just the control group, this difference is also significant ($P=.017$).

Total plasma IgG and IgM levels were determined by ELISA in order to verify whether overall antibody production varied between children with autism (both classic and regressive), controls, and children with Tourette's syndrome. We ran the SAS GLM procedure and found that there was a significant age effect ($F=11.35$, $P=0.001$) on antibody titers for IgG and there was a significant gender effect ($F=9.99$, $P<0.05$) on antibody titers for IgM. There were again no interaction effects of covariates. Using the residuals from the regression analysis controlling for the effects of the covariates, we found that there were no significant differences between the four groups for IgG or IgM (Table 2).

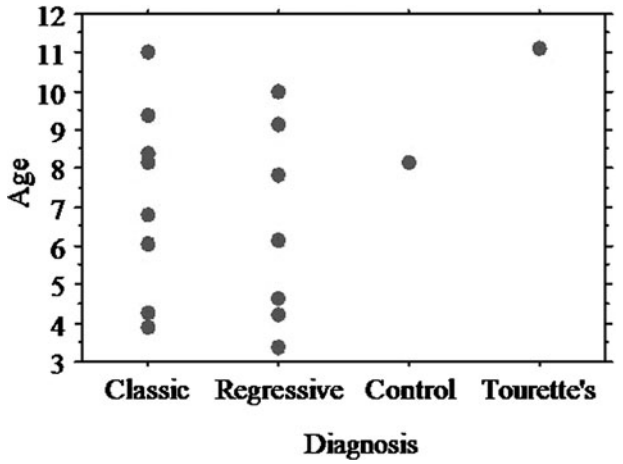

Figure 1 A comparison of age versus diagnosis for 15/59 autism subjects (classic and regressive onset combined) and 2/49 control/Tourette's subjects with little or no antibody titer against rubella virus.

Discussion

Most of the previous studies failing to find a connection between measles and autism are epidemiological in nature, whereas studies finding a connection used biological methods. Therefore, we used biological methods in an effort to clarify this controversial issue further. Our finding of equivalent antibody titers against measles among our four study groups is in agreement with the large body of epidemiological evidence (Smeeth *et al*, 2004; Mäkelä *et al*, 2002; Madsen *et al*, 2002; Taylor *et al*, 1999, 2002; Fombonne and Chakrabarti, 2001; Dales *et al*, 2001; Kaye *et al*, 2001; Peltola *et al*, 1998; reviewed in Madsen and Vestergaard, 2004) and an earlier study of measles antibodies in autism (Peterson and Torrey, 1976) that find no evidence linking measles or MMR with autism.

Previous ELISA studies have found elevated antibody titers against measles or the MMR vaccine in the blood of children with autism compared to controls (Singh *et al*, 2002; Singh and Jensen, 2003). Our sample size was comparable to the samples size used in the Singh *et al* (2002) study, which included 24 children with autism (classic and regressive not distinguished), 14 normal controls, and 16 control children with other disorders. With our sample sizes, we should have been able to detect differences as large as those reported by Singh *et al* (2002) with 87% power (Borenstein *et al*, 2000). Likewise, we should have been able to detect differences as large as those reported by Singh and Jensen (2003), who used 87 children with autism (classic and regressive not distinguished), 32 normal controls, and 14 siblings, with over 85% power (Borenstein *et al*, 2000). The mean age of the study subjects is not reported in either of these studies and could be a confounding factor. Measles blood antibody titers initially increase following MMR vaccination and then decline with age, especially in young children (King *et al*, 1993). Our results were consistent with measles antibody titers significantly decreasing with increasing age. Therefore, when measuring measles antibody titers in blood it appears important to control for age. In the present study, we controlled for age and other factors such as gender and medication status and found no group differences in blood antibody titers to measles virus, rubella virus, mumps virus, or diphtheria toxoid.

In persistent viral infection, antiviral antibody titers are generally high. For example, in persistent measles virus infection, subacute sclerosing panencephalitis (SSPE), antimeasles virus antibody titers are extremely high in the serum and in cerebral spinal fluid (CSF) (reviewed in Asher, 1991; Garg, 2002). One would expect that, if measles virus were persisting in individuals with autism, antibodies to measles virus would be enhanced. This appears not to be the case.

There has been considerable interest, particularly among researchers studying multiple sclerosis, concerning the possible cross-reactivity of antibodies against measles and myelin basic protein (MBP) (Panitch *et al*, 1980). Helping drive this interest is the detection of homologous peptides between MBP and measles virus, among other viruses (Jahnke *et al*, 1985). In those children with autism with antibodies against measles, there has been reported an association with increased sera autoantibodies to MBP (Singh *et al*, 1998, 2002). However, multiple studies fail to find evidence for cross-reactivity of antibodies between MBP and measles virus (Bernard *et al*, 1983; Rubio and Cuesta, 1989; Jingwu *et al*, 1991). Furthermore, when we used the measles titer data (residuals after regression analysis) from the present study and the MBP titer data (residuals after regression analysis) from a previous study (Libbey *et al*, 2007, in press), both of which used the same

Table 2 ANOVA statistical results for Ig concentrations

	Classic	Regressive	Control	Tourette's	*F* value	*P* value
IgG (mg/dl)[1]	546 ± 221 (33)	663 ± 262 (26)	582 ± 335 (25)	634 ± 322 (23)	0.97	.41
IgM (mg/dl)[2]	75 ± 40 (33)	70 ± 53 (26)	92 ± 46 (25)	87 ± 51 (24)	1.15	.33

Ig = immunoglobulin.
[1]Mean mg/dl, adjusted for age ± standard deviations (sample number).
[2]Mean mg/dl, adjusted for gender ± standard deviations (sample number).

patient samples, we found no evidence for a correlation between antibodies against measles and MBP.

Congenital rubella virus infection has long been linked to the development of autism (Libbey *et al*, 2005). In an initial study (Chess, 1971) and a longitudinal follow-up study (Chess, 1977), it was reported that children who had congenital rubella had an increased incidence of autism, compared to the general child population, with a prevalence rate of 741 children with autism per 10,000 children having congenital rubella, suggesting that congenital rubella could be the cause of autism in some children. In addition, an early study by Stubbs (1976) demonstrated that 5/13 children with autism had undetectable titers to rubella virus despite previous vaccination against rubella, whereas all six of the control children who had received previous rubella vaccination had detectable antibody titers against rubella virus. Our finding of a significant number (15/59) of autism subjects (classic and regressive combined) that had either very low or no antibody titer against rubella virus, compared to a combined control/Tourette's group or control alone, supports the findings of Stubbs (1976). With the exception of the one subject with Tourette's syndrome, all subjects received at least one, and most received two, vaccinations. Therefore, the absence of antibody against rubella in these subjects cannot be explained by a lack of vaccination. Of the 15 autism subjects, 2 were female and 13 were male; 3 had colds, 1 had influenza, 3 suffered from allergies, and the rest were not ill at the time of the blood draw; and only 4 subjects were on drugs, other than a multivitamin, including (a) melatonin, (b) Zyrtec, (c) a megavitamin, zinc, and omega-3, and (d) Metadate CD, trazadone, Tylenol, and slow-release iron, on the day of the blood draw. So the factors of gender, illness, and drug use do not correlate with the lack of antibody against rubella. This lack of antibody response may indicate an altered immune responsiveness to rubella in autism. We are currently investigating the T-cell response to rubella in these subjects as a means of determining whether a decrease in helper T cells may explain the lack of antibody response.

Our primary purpose for measuring total plasma IgG and IgM levels was to determine if levels of these antibodies could account for any group differences in antibody titers to pathogens. Previous studies of blood IgG levels in autism are discordant with some researchers finding levels high (Ferrari *et al*, 1988; Croonenberghs *et al*, 2002), low (Zimmerman *et al*, 1995) or normal (Peterson and Torrey, 1976; Plioplys *et al*, 1994; Plioplys, 1998). Our findings are in agreement with the latter group. Most previous studies agree that IgM levels are normal in autism (Peterson and Torrey, 1976; Plioplys *et al*, 1994; Plioplys, 1998; Croonenberghs *et al*, 2002), although one study finds evidence that levels are increased in some cases of autism (Gupta *et al*, 1996). We also find normal levels of IgM.

In conclusion, we found no evidence of an increase in the level of antibodies against measles virus in autism subjects, compared to controls, thus, further disputing the claim that measles vaccine or infections cause autism. Likewise, no correlation was found between antibodies against measles and autoantibodies to MBP in autism. There were, however, a significant number of children with autism that had an impaired ability to respond to rubella virus. This needs to be explored further.

Materials and methods

Subjects and design

A description of the subjects and the design of the experimental study can be found elsewhere (Kirkman *et al*, 2007, in press). All consent forms and approvals were obtained. Participants were recruited from the Child and Adolescent Psychiatry Clinics at the University of Utah, School of Medicine, and from the surrounding community. All subjects met the *Diagnostic and Statistical Manual of Mental Disorders* (Fourth Edition) (DSM-IV) (American Psychiatric Association, 2000) criteria for autism or Tourette's syndrome as determined by a board-certified child and adolescent psychiatrist or psychologist. To verify correct diagnosis, autism subjects, as well as controls, were administered the Autism Diagnostic Observation Schedule—Generic (ADOS-G) (Lord *et al*, 2000). Control subjects who showed any significant social or communicative concerns on the ADOS-G were excluded from the study. Subjects with autism were also administered the Autism Diagnostic Interview—Revised (ADI-R) (Lord *et al*, 1994). Specific questions in the ADI-R are designed to determine regression status (Goldberg *et al*, 2003; Luyster *et al*, 2005). We tried to be as inclusive as possible with our definition of regression, as regression may occur at a variety of ages and involve a wide range of presentations. Subjects had no major diseases other than autism or Tourette's syndrome and were relatively healthy (colds and flu-like symptoms, ear infections, asthma, and allergies accepted). Subjects were not taking either selective serotonin reuptake inhibitors (SSRIs) or the serotonin receptor antagonist, risperidone (Schotte *et al*, 1993). Medications were coded into five groups, psychiatric medicines, stimulants, anti-inflammatory medicines, antibiotics, and other drugs, for the purpose of regression analysis as described previously (Kirkman *et al*, 2007, in press).

Blood was drawn and centrifuged to obtain plasma prior to freezing at -70°C. Plasma from children with autism, controls and Tourette's syndrome were compared for antibodies to measles virus, rubella virus, diphtheria toxoid, mumps virus, and total levels of IgG and IgM. The subject groups were of similar age and gender composition, except for the significantly older Tourette's group due to the older age of onset of Tourette's. Measles virus, rubella virus, mumps virus,

and diphtheria toxoid were used to determine antibody responses to vaccines or wild-type infections. Total IgG and IgM levels were determined to verify if overall antibody production varied between the groups. Antibody levels were determined by ELISA. Prior to further analysis, the antibody titers were adjusted using regression for significant effects of covariates. Covariates were dropped if they did not have significant main or interaction effects on antibody titer. All technicians performing the ELISAs were blinded as to the diagnosis of the individual samples tested.

Antigen preparation
Measles, rubella, and mumps viruses: Vero cells (American Type Culture Collection [ATCC], Rockville, MD) were infected with measles virus (Edmonston strain, ATCC), rubella virus (RA27/3 strain, ATCC), mumps virus (Enders strain, ATCC), or mock infected, as described previously (Fujinami and Oldstone, 1980). Infected cells underwent rapid freeze/thaw and sonication prior to partial purification by centrifugation. Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's recommendations.

Diphtheria toxoid: Diphtheria toxoid was purchased from List Biological Laboratories (Campbell, CA).

ELISA
Total IgG and IgM values were determined by ELISA quantitation kits purchased from Bethyl Laboratories (Montgomery, TX), according to the manufacturer's recommendations. For all other antigens, ELISAs were performed as previously described with some modification (Rice and Fujinami, 1986). Flat-bottomed 96-well Nunc-Immuno plates, MaxiSorp surface (Nalge Nunc, Rochester, NY) were coated overnight at 4°C, with 10 μg/ml antigen solution in phosphate-buffered saline (PBS). Plates were washed with PBS containing 0.2% Tween-20 prior to blocking with PBS containing 0.2% Tween-20 plus either 5% fetal calf serum (FCS) (diphtheria) or 10% FCS (measles, rubella, mumps) for 60 min. The blocking solutions were used as the diluent throughout the assays. Plasma were serially diluted twofold across the plates, starting at concentrations as low as 1:64 (2^6), and incubated at room temperature for 90 min. After washing 50 μl of horseradish peroxidase–conjugated goat anti-human IgG (Jackson Immuno Laboratories, West Grove, PA) was added to each well at a dilution of 1:5,000, or for the diphtheria toxoid assay, 1:10,000. After a final wash reaction, products were visualized by adding 50 μl/well of substrate containing *o*-phenylenediamine dihydrochloride (Sigma, St. Louis, MO). The color was allowed to develop for 30 min in the dark and stopped by addition of 50 μl/well 1 M HCl. Wells were quantitated as absorbance at 492 nm on a Titertek Multiscan Plus microplate reader (Titertek Instruments, Huntsville, AL). All measurements were performed in duplicate. Control wells that received no plasma were used as background values. All data were corrected by subtraction of these background values. For the measles, mumps, and rubella assays, subjects' plasma were tested in wells coated with just mock-infected Vero cell extracts, and the optical densities from these wells were subtracted from the values of similarly treated wells coated with virus-infected Vero cell extract. Intraassay coefficients of variance were determined by running a control sample multiple times within a plate. A control sample was repeatedly run on various plates throughout each assay for use in determining interassay coefficients of variance. Optical density values (reported as: optical density $\times$ 1000) are reported at plasma dilutions where standard curve readings were linear and the coefficients of variance were optimal.

Statistical analysis
The StatView or SAS programs (SAS Institute, Cary, NC) were used to perform all statistical analyses. Group differences for continuous data were determined by ANOVA, followed when indicated by the Fisher's protected least significant difference (Fisher's PLSD) post hoc test. For nominal data such as gender, we utilized the chi-square test. After checking for basic group differences, the SAS GLM procedure was used to generate residual ELISA scores adjusting for age, gender, and medication status. Subsequent analyses of the ELISA results used these residual scores. We did not find significance when testing for differential effects of covariates within diagnostic group and within gender. The entire sample was therefore used for covariate adjustment, rather than adjusting within diagnosis or within gender. The Power and Precision program (Borenstein *et al*, 2000) was used to determine statistical power.

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