Detection of Measles Virus Genomic RNA in Cerebrospinal Fluid of Children with Regressive Autism: a Report of Three Cases

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ABSTRACT

In light of encephalopathy presenting as autistic regression (autistic encephalopathy, AE) closely following measles-mumps-rubella (MMR) vaccination, three children underwent cerebrospinal fluid (CSF) assessments including studies for measles virus (MV). All three children had concomitant onset of gastrointestinal (GI) symptoms and had already had MV genomic RNA detected in biopsies of ileal lymphoid nodular hyperplasia (LNH).

Presence of MV Fusion (F) gene was examined by TaqMan real-time quantitative polymerase chain reaction (RT-PCR) in cases and control CSF samples. The latter were obtained from three non-autistic MMR-vaccinated children with indwelling shunts for hydrocephalus. None of the cases or controls had a history of measles exposure other than MMR vaccination. Serum and CSF samples were also evaluated for antibodies to MV and myelin basic protein (MBP).

MV F gene was present in CSF from all three cases, but not in controls. Genome copy number ranged from 3.7×10^4 to 2.42×10^7 per ng of RNA total. Serum anti-MBP autoantibodies were detected in all children with AE. Anti-MBP and MV antibodies were detected in the CSF of two cases, while the third child had neither anti-MBP nor MV antibodies detected in his CSF.

Findings are consistent with both an MV etiology for the AE and active viral replication in these children. They further indicate the possibility of a virally driven cerebral immunopathology in some cases of regressive autism.

Background

A possible association between MMR vaccination and autistic encephalopathic regression (AE) in previously developmentally normal children has been reported.[1-5] Evidence for this link is controversial, reflecting the widely differing conclusions of basic and clinical science[1-5] vs. epidemiology,[6-10] although the association is supported by a recent report based upon the CDC's Vaccine Adverse Events Reporting System (VAERS).[11]

Measles, mumps, and rubella viruses, in their natural form, have been linked to childhood developmental disorders including autistic spectrum disorder (ASD),[12-14] disintegrative disorder,[15] and developmental regression.[16] Deykin and MacMahon compared exposure patterns of 183 children with autism and 355 sibling controls to the encephalitogenic viruses, measles, mumps, rubella, and chickenpox.[12] They found that autistic manifestations were associated with prenatal experience with measles and mumps. Ring et al., using statistical modelling of the number of autism births compared with epidemics of measles, rubella, poliomyelitis, viral meningitis, and viral encephalitis in Israel, found that children born during epidemics of measles were at greater risk of developing autism.[13]

Pathogenetic studies of children with regressive autism and gastrointestinal (GI) symptoms have identified an intestinal mucosal lesion that is consistent with a viral etiology.[17-20] The salient features include ileocolonic lymphodular hyperplasia (LNH) and a patchy, panenteric mucosal immunopathology characterized by an increased lymphocyte density, predominantly of $CD3^+$, $CD8^+$, and $CD19^+$ phenoytpes. Flow cytometric analysis of mucosal lymphocyte intracellular cytokine profiles has identified extensive immunodysregulation in affected children, characterized by a significant excess of tumor necrosis factor alpha (TNF-α), raised interferon gamma (INF-γ), and a reduced counter-regulatory interleukin-10 (IL-10), in biopsies from duodenum, ileum, and colon.[21] The data are consistent with evidence of systemic up-regulation of proinflammatory cytokines in similarly affected children.[22] The findings are reminiscent of human immunodeficiency virus (HIV) enteropathy, which has been reviewed by Schneider et al.[23] and Zeitz.[24]

Uhlmann et al. have reported the presence of measles virus (MV) genomic RNA in the hyperplastic ileal lymphoid tissues of affected children at significantly higher prevalence than in developmentally normal controls.[3] MV nucleocapsid (N) protein has been identified in the same location.[25] Singh et al. have reported atypical humoral immune responses to MV in children with a similar autistic presentation.[2,4] Serum anti-MV, but not rubella and mumps virus, immunoglobulin G (IgG) antibody levels were significantly higher in children with AE than in developmentally normal children of the same age, including siblings of autistic children. MV IgG titers exhibited a significant positive correlation with serum antibody titers to MBP. In addition, specific IgG antibody to a 74kD protein extracted from MMR vaccine, and consistent with MV Hemagglutinin (H) protein, was identified in 80% of affected children, but not in control sera.

These findings raise the possibility that any potential etiologic link between MV and AE may operate through at least one of three non-mutually exclusive mechanisms. These include direct viral invasion of the brain by this neurotropic virus, cerebral autoimmunity with or without the presence of intracerebral MV as in post-measles encephalomyelitis, and an indirect gut-brain interaction mediated by a toxic encephalopathy in a manner analogous to hepatic encephalopathy.[26]

This report on three children with both AE and intestinal pathology associated with MV persistence describes for the first time detection of MV genomic RNA in the CSF of such children. The data support the growing perception that a subset of children with AE exhibits a complex systemic pathology consistent with an etiological role for MV.

Patients and Methods

Children with AE were selected for reporting based upon clinical criteria of normal early development for at least the first year of life, neurologic deterioration associated with autistic regression indicating the need for lumbar puncture, and GI symptoms necessitating ileocolonoscopy.

In addition, cerebrospinal fluid (CSF) and ileal lymphoid tissue from all three children had been submitted for analysis for MV genomic RNA. They represent the first three children to complete this testing among a much larger cohort of similarly affected children under the care of two of the authors (JB & JK). All the children are male Caucasian. None of these children are included in previously published work on the subject by Wakefield and colleagues. All have received multiple interventions ranging from dietary modification to intravenous immunoglobulin, though the details of these interventions and the relevant outcomes are not reported here. All three children in this study received the MMR II (Measles, Mumps, and Rubella Virus Vaccine Live), Merck & Co., Inc., hereinafter MMR II.

Child 1*:* This child presents after a full-term, normal delivery with no relevant prior medical history and normal development to 14 months of age. Following MMR II vaccination given with varicella vaccine (Varivax), he deteriorated progressively, with onset of ataxia, loss of interest in parents and surroundings, altered pain threshold and sleep pattern, self-limited diet, and explosive diarrhea with poor weight gain. There was a strong family history of autoimmunity, and thyroid disease in particular. Extensive neurologic, genetic, and metabolic testing was normal. A lymphocytosis was detected in this child. Counts were: $CD3^+$, 3071 cells/µl (normal range 582-1992); $CD4^+$, 1918 cells/µl (normal range 401-1532); $CD8^+$, 990 cells/µl (normal range 152-838); and $CD19^+$, 1389 cells/µl (normal range 71-567).

He underwent upper GI endoscopy at 3.5 years of age. Findings included mild esophagitis with normal gastric and duodenal histology. Lactase, maltase, sucrase, palatinase, and glucamylase activities were normal, and serum anti-endomyseal antibodies were negative. Ileocolonoscopy showed ileal LNH, and histology revealed focal acute ileitis with cryptitis and crypt abscess formation. Routine stool cultures and microscopy for ova and parasites were negative.

Lumbar puncture was performed at age 3 years, 7 months. Clear spinal fluid was obtained. Routine CSF analyses including protein, glucose, and cell counts were within normal limits, and bacterial culture was negative.

Child 2: The second child presents after a full-term, normal delivery with a medical history of intolerance to formula food, and recurrent upper respiratory tract and ear infections, necessitating myringotomy and adenoidectomy in the first year of life. He developed normally to 15 months of age. He received his first MMR II vaccination, with *Hemophilus influenzae* serotype b (Hib) conjugate vaccine, at 15 months of age. By 18 months of age his parents noted decreased sociability, loss of pain perception, repetitive behaviors, and increasing agitation and inattention, associated with the onset of chronic diarrhea and weight loss.

Following a booster MMR II vaccine at age 4, he suffered subacute neurologic deterioration with onset of petit mal seizures, which necessitated neurologic investigation within six weeks of his booster MMR II. This confirmed AE with seizure disorder, with a grade II dysrhythmia on EEG. MRI scan was normal. The child was lymphopenic, including a deficiency in $CD3^+$, with 1082 cells/µl (normal range 2040-6000); $CD4^+$, with 592 cells/µl (normal range 1300-4100); $CD8^+$, with 346 cells/µl (normal range 750-2600), and $CD19^+$, with 216 cells/µl (normal range 610-1830).

He underwent upper GI endoscopy and ileocolonoscopy at age 6 years, 6 months for chronic diarrhea. He had moderate ileal LNH but was otherwise endoscopically normal. Histology was normal other than a mild patchy lymphocyte infiltrate in the gastric mucosa. He had serologic evidence of past infection with *H. pylori*, but no evidence of this organism on gastric histology. Routine serology, stool cultures and microscopy, and celiac screen were otherwise normal.

Because of his encephalopathy, lumbar puncture was performed at age 8 in light of the ability to detect MV in intestinal tissues from some autism spectrum disorder (ASD) children, combined with the detection, in his serum, of antibodies to MBP and neuron-axon filament protein (NAFP). At lumbar puncture, clear fluid was obtained. Routine CSF analyses including protein and cell counts were within normal limits, although glucose was marginally raised at 72 mg/dl (normal range 40-70). Bacterial culture was negative.

Child 3: Child 3 presents after a full-term, normal delivery with no relevant prior medical history and normal development to 16 months of age, when he received MMR II and Hib vaccines while being treated with sulfamethoxazole-trimethoprim (Bactrim). Thirteen days later he returned to the doctor's office with a severe upper respiratory tract infection, which became chronic and

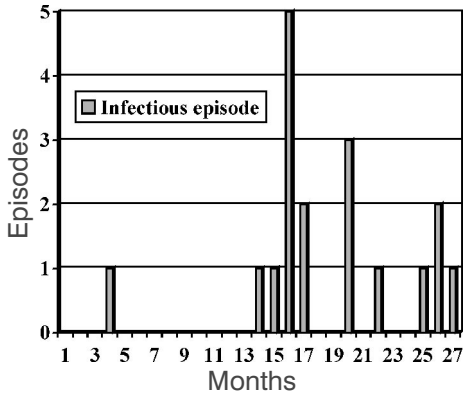

Figure 1. Infectious Episodes Before and After MMR
Number of infectious episodes recorded for child 3 during the first 27 months of life, providing a comparable length of time before and after MMR vaccination at 16 months. Infectious episodes, diagnosed by a physician and documented in the medical records, total 1 per 5-6 months before MMR, 1 per month afterwards.

refractory to antibiotic therapy (Figure 1). Within about 30 days of the MMR II, he was hospitalized for dehydration, mental status changes, and high fever.

From this point on, he suffered chronic diarrhea and weight loss, dropping from the 50th to the 10th percentile for weight in two months. Eye contact was lost within two weeks of MMR II. Loss of speech occurred from 18 months of age. Owing to recurrent fevers and failure to thrive, a rheumatologist examined him at 17 months and made a provisional diagnosis of juvenile rheumatoid arthritis (RA). Further investigation was not undertaken. A neurologic assessment at age 2 by a previous pediatrician noted no speech, no eye contact, aberrant behavior, and decreased strength on the right side. Despite these observations, no tests were conducted. An EEG performed at age 5 years, 6 months was reported as normal.

Ileocolonoscopy and upper GI endoscopy were performed at age 4 years, 6 months. Findings included ileal LNH with petechial hemorrhage (Figure 2). A mucosal eosinophilic infiltrate was observed in esophageal, gastric, duodenal, ileal, and cecal biopsies. This infiltrate ranged from >50 eosinophils per high power field HPF in the duodenum, to approximately 150 per HPF in the ileum. In addition, mild chronic inflammation was observed in the cecal biopsy. Screening for *H. pylori*, rotavirus, and stool pathogens including ova and parasites, was negative. Radio-allergosorbent test (RAST) for dietary allergens was positive for wheat, eggs, and milk. Consistent with an allergic predisposition, serum IgE was raised at 2471 IU/ml (normal range 0-164), and IgA was low at 24.9 mg/dl (normal range 36-163).

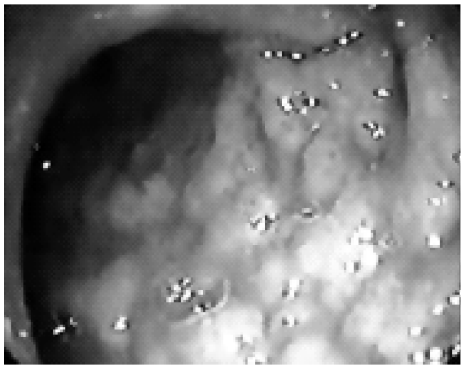

Figure 2. Ileal LNH with Petechial Hemorrhage from Child 3

Serum anti-MBP level was 46 EIA units prior to immunologic intervention. This autoantibody decreased to normal levels (< 10 EIA units) following intravenous human immunoglobulin (IVIG) treatments, but after treatment was suspended, it again rose in association with further decline in cognitive function. Once IVIG was resumed, the serum anti-MBP returned to normal and was normal at the time of CSF acquisition.

Lumbar puncture was performed at age 3 years, 7 months. Clear spinal fluid was obtained. Routine CSF analyses including protein, glucose, and cell counts were within normal limits, and bacterial culture was negative.

Processing of Intestinal Biopsies

GI endoscopy was indicated because of relevant symptoms in these children. All procedures were undertaken with fully informed written parental consent. All procedures were performed under general anesthesia and were uneventful. At ileocolonoscopy, tissue samples were taken from areas of terminal ileal LNH, present in all three children, and from various sites throughout the colorectum. Samples for routine histopathology were placed in 10% neutral buffered formalin and embedded in paraffin wax. Samples of ileal lymphoid tissue for MV analysis were transferred directly from the biopsy forceps into a sterile plastic container, which was sealed, labeled, immediately frozen, and maintained unopened, at -70^{o}C or below, until MV analysis.

All GI mucosal biopsies were submitted for histologic analysis by the same pathologist (AA) and reported systematically, using a standard proforma, as described and validated in blinded review elsewhere.[17] Histologic analysis was conducted in the absence of any knowledge of vaccination status and any results of MV testing on biopsy tissue, CSF, and blood.

Serum and Peripheral Blood Mononuclear Cells (PBMC)

Blood was taken by standard venipuncture. Serum samples for antibody analysis were taken in Serum Separator Tubes (SST, Becton Dickinson, NJ). Whole blood for viral analysis was taken in EDTA anticoagulant. All samples were shipped on dry ice.

Lumbar Puncture

In the AE children, CSF analyses were considered indicated in the presence of an undiagnosed regressive encephalopathy following viral exposure. CSF was obtained with informed parental consent. Since informed consent had been granted for diagnostic purposes, IRB approval was not required for the procedures or laboratory testing. The Arizona State University IRB granted ethical approval for retrospective evaluation of the ICDRC data.

Spinal fluid was obtained after intravenous conscious sedation and local anesthesia, using sterile technique. The fluid was collected in three standard CSF tubes using a commercial kit (Portex, Inc., Keene, N.H.). A minimum of 4 cc of CSF was obtained from each child. Two ml samples for TaqMan real-time quantitative polymerase chain reaction (RT PCR) were labeled and incubated overnight at 2°C in RNA-Later (Ambion, Austin, Texas) according to manufacturer's instructions, then frozen at -70° C. Samples were maintained unopened and on dry ice until analyzed.

Control CSF

Three pediatric CSF control samples were provided by Tulane University Medical Center. Samples came from children requiring CSF shunts for hydrocephalus who did not have autism. They included a girl aged 11 with von Hippel-Lindau disease, a boy aged 10 with Hunter's syndrome, and a girl aged 2 with an ependymoma. All three children were up to date with their MMR vaccination, as per the normal U.S. vaccination schedule.

Samples were collected and stored in the same manner as the CSF samples from AE children. Control samples were coded and batched with other coded samples prior to shipping on dry ice to the analytical laboratory, where they were tested in a blinded fashion. The Tulane University Medical Center IRB Committee granted ethical approval.

Positive Controls

Biological Controls: A brain tissue sample from a confirmed case of subacute sclerosing panencephalitis (SSPE) was used as a positive control. This tissue was examined by solution phase, in cell, and TaqMan RT-PCR and strain-specific determination by traditional sequencing using dideoxy sequencing and Allelic Discrimination (AD) assay. A further postmortem brain tissue control was used from a child with post-MMR vaccine encephalitis. This sample was analyzed by solution phase, in cell and TaqMan RT-PCR, and AD assay.

Negative Controls

Negative samples included paraffin-embedded tissue from appendix, thyroid, and breast; fresh cell culture TPC-1, N-thy, and Raji cells; buccal swabs; and PBMC from operator and random blood samples. Environmental controls, including water collected from various locations in the laboratory, were included to rule out the possibility that positive results were due to airborne contamination in the lab.

RNA Extraction

Total RNA was extracted from CSF, fresh frozen ileal biopsies, and PBMC using the Qiagen RNeasy (CSF) (QIAGEN Ltd, Crawley, West Sussex, U.K.) and Ultraspec-11 RNA isolation system (biopsies and PBMC) (Biotecx Laboratories, Texas, U.S.). Environmental controls were extracted in the same manner as CSF, and tissue controls were extracted in the same manner as biopsies and PBMC.

TaqMan RT-PCR

RT-PCR based on the 5' nuclease assay was performed on an ABI 7700 Sequence detector (Applied Biosystems) as described previously.[3] Sequence-specific PCR primers and TaqMan probes were designed using Primer Express software (Table 1).[3] All quantitative PCRs were prepared in a dedicated facility in a class 2 laminar flow bench, using dedicated pipettors and aerosol resistant pipette tips as described by Uhlmann et al.[3] Controls for TaqMan RT-PCR included the following: no template control (water added as template), no amplification control (omission of rTth polymerase), irrelevant target primers and specific TaqMan probe (human papillomavirus 16, human herpes virus 8 primers), probe-only control (omit PCR primers), human RNA control, spiked RNA control, and asymmetric TaqMan PCR (TaqMan PCR with one or other primer and specific TaqMan probe).

Table 1. Measles Virus Primer and Probe Sequences

Primer/ Probe	Sequence 5'- 3'	Sequence 5'- 3'
F1 Fwdc	TGA CTC GTT CCA GCC ATC AA	
F1 Rev c	TGG GTC ATT GCA TTA AGT GCA	150BP
GAPDH 1	GAA GGT GAA GGT CGG AGT	
GAPDH 2	GAA GAT GGT GAT GGG ATT TC	226BP
F1 Probe	CTG CAC GAG GGT AGA GAT CGC AGA ATA CAG	
GAPDH Probe	CCG ACT CTT GCC CTT CGA AC	

A gene dosage correction for bowel biopsies and PBMC was carried out using glyceraldehyde phosphate dehydrogenase (GAPDH) as a housekeeping gene. MV quantitative TaqMan RT-PCR was performed by generating standard curves for the F gene. This was achieved using serial dilutions of cloned cRNA transcripts over a linear dynamic range. Cloning of the cRNA standards was performed using the TOPO TA cloning system, according to the manufacturer's instructions (Invitrogen, Groningen, Netherlands).

Viral Antibody Detection

Viral antibodies were measured by using commercially available enzyme-linked immunoabsorbent assay (ELISA) kits (Sigma Diagnostics, St. Louis, Mo.). Assays were performed essentially according to technical instructions of the manufacturer of the ELISA kits. Subsequently, the antigenic detection of measles virus was attempted by immuno-blotting that was performed according to Singh.[4]

Viral proteins were separated in 12% Ready Gels (Bio-Rad Labs, Richmond, CA) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). They were transferred to

Table 2. TaqMan RT-PCR Detection of MV F Gene in Ileal Lymphoid Tissue, Peripheral Blood and CSF

ASD Child	Ileal Biopsy		Blood		CSF	
	+ve MV RNA	MV F Gene Copies / ng Total RNA	+ve MV RNA	MV F Gene Copies / ng Total RNA	+ve MV RNA	MV F Gene Copies
1	Y	<1	ND	-	Y	$2.42x10^7$
2	Y	$1x10^3$	Y	2.1	Y	$6.16x10^6$
3	Y	>7	N	0	Y	$3.7x10^4$
Control Child	**Ileal Biopsy**		**Blood**		**CSF**	
	+ve MV RNA	MV F Gene Copies / ng Total RNA	+ve MV RNA	MV F Gene Copies / ng Total RNA	+ve MV RNA	MV F Gene Copies / ng Total RNA
1	-	-	-	-	N	-
2	-	-	-	-	N	-
3	-	-	-	-	N	-

nitrocellulose membranes (NCM) by double sandwich technique, followed by blocking with 1% bovine serum albumin in tris-buffered saline (TBS). The NCMs were stored at room temperature.

For immunoassay, 3-4 mm wide blots were cut and incubated with human sera for one hour. After four washings with TBST (TBS buffer containing 0.05% Tween-20), the blots were incubated for one hour with goat anti-human polyvalent-alkaline phosphatase conjugate. After four washings, the blots were developed in substrate solution by using the technique described by Singh.[4] A reaction was scored positive whenever a purplish-blue band was seen.

Brain Antibody Detection

Brain autoantibodies to myelin basic protein (anti-MBP) and neuronal filament protein (anti-NAFP) were detected by an immunoblotting method, essentially according to published reports by Singh et al.[5] Briefly, the proteins (bovine myelin basic protein from Upstate Biotechnology Inc., Lake Placid, N.Y.) and bovine spinal cord neurofilament protein preparation from Singh's ongoing work in this area were separated in 12% polyacrylamide Ready Mini-Gels (Bio-Rad Labs, Richmond, Calif.) under the denaturing conditions of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol. The gels were run at 150 V for about 45 minutes, and protein transfer was achieved by a double-sandwich technique for more than 20 hours at room temperature. When possible, confirmation studies were performed at Specialty Laboratories, Inc., a Santa Monica, Calif., commercial lab. Both Dr. Singh and Specialty Laboratories were able to detect anti-MBP at the same rate.

Results

TaqMan Detection of MV F Gene

All three ileal biopsy and CSF samples from subject children were positive for MV Fusion (F) gene by TaqMan RT-PCR. The three control samples from non-AE children were negative for MV F gene. All negative controls including process controls and tissue controls, as described above, run in parallel with samples from AE and control children, were also negative for MV F gene. Of the PBMC samples available for two children, one was positive and one was negative for MV F gene. Details of results and F gene quantification are in Table 2.

Positive Tissue Controls

Brain tissue samples from the SSPE and post-MMR vaccine encephalitis cases were all positive for MV by solution phase, in cell, and TaqMan RT-PCR.

Antibody Studies

Results of CNS autoantibody and virus IgG profiling are shown in Table 3. MBP autoantibodies were present in the serum of all three children and CSF of children 1 and 2. NFAP antibody was present in the serum of child 2 only. MV IgG antibody titers were reported as high in the sera of children 1 and 2, and detectable at a low level in the CSF of these same children. MV IgG antibody titer was reported as being within the normal range in the serum of child 3 and undetectable in his CSF. Where samples were analyzed for the previously reported MMR-associated antibody,[4] they were negative. Human Herpesvirus-6 serology was unremarkable, and specific IgG antibody was not detected in CSF of the two samples in which it was sought (children 2 and 3).

Discussion

This study reports for the first time simultaneous detection of MV genomic RNA in at least two sites–ileal lymphoid tissue and CSF–in three children with regressive autism. Presence of MV genomic RNA in CSF is associated with the presence of MV IgG

Table 3. Serum and CSF Antibodies

ASD Child	Sample	Myelin Basic Protein Antibody*	Neuron-Axon Filament Protein Antibody**	MV IgG Antibody#	MMR Antibody##	Human Herpesvirus-6 IgG Antibody*#
1	Serum	Y	N	Y (4.51U)	N	Y (1.39U)
	CSF	Y	N	Y (0.5U)	N	ND
2	Serum	Y	Y	Y 355 EIA U***	ND	ND
	CSF	Y	N	Y (0.4U)	N	N
3	Serum	Y^	N	Y (3.93U)	ND	Y (1.97U)
	CSF	N	N	N	N	N

Y=positive, N=negative, ND=not done.
Serum MBP positive at dilution of 1:400, at which normal serum is negative. NAFP dilution >1:50 MV seropositive at > 1.1 ELISA units (U).[4,5] Reference range 3.1+0.1 U. HHV6 seropositive at >1.1. Reference range 1.5+0.1 U. *Screened at 1:26 dilution in CSF; ** Screened at 1:26 dilution in CSF; #Screened at 1:5 dilution in CSF; ##Screened at 1:5 dilution; *#Screened at 1:8 dilution. The specific units in Singh's work are based on the Sigma kit and are EIA MV IgG.[4-5] ***In relationship to child 3, several anti-MBP titers were drawn, and they ranged from a high of 46 EIA (strongly positive) units prior to intervention to a low of 4 EIA (within normal limits) units after IVIG intervention. ^These results were performed and verified at Specialty Laboratories of Santa Monica, California. All other antibody studies were performed by Dr. Singh at Utah State University.

and anti-MBP antibody in CSF in two of these children. MV genomic RNA was not detected in CSF from non-AE children.

There may be several explanations for these findings. First, detection of MV could represent laboratory contamination. However, the absence of MV in any process control run in parallel with the test samples and the absence of MV in control CSF strongly militate against this. Sources of potential contamination have been investigated and excluded: Buccal swabs and blood from technicians were PCR negative. Operator set-up and chemistry contamination was precluded by use of PCR Mastermix. Airborne laboratory contaminants were excluded by environmental laboratory controls. Contamination during the extraction process was excluded by random blood, tissue, cell culture, and operator controls. PCR set-up contamination was excluded by no-template controls. Nonspecific amplification was excluded by irrelevant primer and nonsense primer controls and the presence of negative samples.

Where synthetic oligonucleotide controls were employed, these were synthesized in an overseas facility, remote from the production facility for the primer and probes for that target control. These controls were received in a laboratory located in a separate hospital from that used to set up the TaqMan assay. Controls were diluted to 10^{-10} before transfer to the testing laboratory. They were stored at this dilution in a separate freezer from samples, primers, or other reagents.

Presence of MV in the CSF may not necessarily reflect intracerebral persistence of this agent. Experience with HIV infection has shown, by using exquisitely sensitive detection techniques such as TaqMan, that viral genomic material may be amplified from infected immune cells as they traffic through the CSF in the absence of obvious neurological abnormalities or parenchymal infection of the brain (reviewed by Tyler and McArthur).[27] This possibly explains AE children who harbor a lymphotropic virus such as MV in peripheral lymphoid tissues, e.g. the intestinal mucosa. However, the low cell counts in the CSF (all less than five white blood cells per HPF) would indicate a highly improbable relationship between trafficking lymphocytes and detection of MV genome.

Additionally, viral genome in the presence of MV IgG in the CSF of two children is supportive of intrathecal antibody synthesis, probably in response to intracerebral infection. This is supported by detection of antibodies to MBP in the CSF of the same children. Intracerebral infection may also occur in the apparent absence of local antibody synthesis.[28] Differences in methodologies used for CSF and sera prevent ratio of sera to CSF calculations. This should be systematically evaluated in future studies.

Vaccinations occurring in close temporal proximity to the encephalopathic regression of these children, when combined with the lack of documented natural MV exposure and a very low endemic MV rate, make it likely that the persistent MV infection originated from the vaccine. The children's relevant clinical symptoms started soon after MMR vaccination (documented as soon as 13 days after exposure in child 3).

The retrospective analysis of this data will not allow for strain-specific determination since that testing was not requested of the laboratory at the time of submission. Further, TaqMan RT PCR does not allow for subsequent strain discrimination by traditional sequencing. However, Sheils et al. have recently reported an allelic discrimination assay that distinguishes Schwarz vaccine-derived strains from all but a few wild-type and laboratory-derived strains.[29] Samples of ileal lymphoid tissue[29] and CSF (Sheils and O'Leary, personal communication, 2004) from other, similarly affected children have all yielded strains consistent with vaccine strain. Therefore, on balance, the persistent virus in AE children is most likely to have originated from the vaccine. If not, a matter of equal concern is that these children would then represent a previously undocumented presentation of atypical wild-type measles following vaccination, i.e. "vaccine failure."

According to the literature, measles vaccine virus may, on rare occasions, cause cerebral infection and neuropathology. Bitnum reported the development of measles inclusion-body encephalitis (MIBE) caused by the measles vaccine strain (as MMR) in one child.[30] Notable characteristics of this case included the unusual delay in onset of neurological symptoms following MV exposure (8.5 months) and the absence of any apparent evidence of pre-existing immunodeficiency. Measles virus itself is well known to be immunosuppressive. There is no evidence of MIBE or SSPE in any of the three cases presented here.

Child 3 developed a new onset of immune dysfunction following MMR exposure. This is consistent with the observations of wild-type measles infection and the ongoing immune disruption caused by viral persistence, rather than prior immune deficiency. Child 1 did have a significant history of infection suggestive of immune dysfunction prior to vaccination, but Child 2 had what were considered routine infections prior to MMR. These differences may represent the various environmental influence on the immune system combined with genomic susceptibilities.

A further explanation of our data might be that the sensitive testing involved is detecting persistence of mere fragments or residues of MV RNA, rather than replicating virus or active subviral elements. This is unlikely for RNA, which is notoriously prone to rapid enzymatic degradation. Permar et al. have indicated that detection of MV genome in two or more body compartments is indicative of continued viral replication.[31] Whether or not this is the case, the detection of viral RNA in PBMC in one child is indicative of replication, given the relatively rapid turnover of circulating immune cells. In addition, previous studies have reported the presence of all MV genes–nucleocapsid (N), hemagglutinin (H), and fusion (F)–that have been sought in ileal lymphoid tissues from ASD children.[3] Studies have also detected the MV N protein by monoclonal and polyclonal antibody

immunohistochemistry.[25] Based upon these observations, it seems more likely than not that MV is replicating in these AE children, albeit at presumably low levels.

The findings are unexpected in view of the negative epidemiologic data from the U.S. and Europe. These retrospective population-based studies, in contrast with molecular and immunological studies, have not found an association between MMR vaccination and autism. As pointed out by Madsen et al.[10] and reiterated by others,[32-34] epidemiologic studies that have examined this relationship have lacked adequate statistical power and have failed to test the correct hypothesis.

Madsen et al. themselves failed to disaggregate the relevant autism subset–one they attempt to describe in their paper's introduction–from the total autism population. This is equivalent to considering hepatitis as a single outcome, irrespective of etiology, in a study designed to examine a possible causal relationship with a single, specific exposure that may account for only a minority of hepatitis cases.

In distinction to the aforementioned studies, and as previously stated, Geier and Geier in evaluating the U.S. VAERS database found a positive association of MMR and autism with an attributable risk of 4.2 ($p < 0.0001$).[11] Adding to the complexity of evaluating this condition from an epidemiologic perspective is the well recognized heterogeneity of this population. Specific hypothesis testing for viral persistence in the CSF in the presence of encephalopathic regression, rather than autism per se, is crucial.

Child 3 presents an interesting array of findings. While both other children demonstrate the presence of both anti-MV and anti-MBP antibodies in the CSF, this third case lacks detectible antibodies in the CSF to either brain or measles virus. This is compatible with various and complex explanations. The immunological interventions prior to acquiring CSF could have altered the outcome of CSF immune findings without changing the viral persistence observed. As noted, serum anti-MBP levels normalized with IVIG.

Further, prior apoptotic immune signalling from primed microglial cells is one theoretical explanation of the findings in child 3, and has been discussed in relationship to Parkinson's disease.[35] Of note are the child's episodically very high levels of peripheral serum anti-MBP. This is a nonspecific finding, but is compatible with the measles etiologic hypothesis based on the classic understanding of the role of MV in CSF disorders.[36] Singh has reported his opinions and observations that cytokines and/or abnormal immune response to the vaccine MV could account for the neurological and immunological findings in AE.[5] It is beyond the scope of this case report to review the literature on all mechanisms of neurodegenerative injury secondary to virus-mediated disorders. However, it is conceivable that multiple mechanisms are active in the children, and that they vary over time based on other factors including concurrent viruses, toxins, and oxidative stressors.

Autistic encephalopathy is a complex disorder in which there is clearly more than one potential mechanism for regression. Cofactors including genetic predisposition are likely to influence the presentation and timing of symptom development. The potential mechanisms of AE in these children include, but are not limited to, some or all of the following: a toxic gut-brain interaction such as occurs in hepatic encephalopathy; immunological disruption of central nervous system functions; and direct viral invasion of the brain.

Preliminary evidence for an autoimmune phenomenon in some AE children reported here and elsewhere, and alternative mechanisms of encephalopathy in these children, require detailed study. These data indicate that sensitive and specific virological and immunological analysis of CSF is indicated in children undergoing AE with regression following exposure to live virus vaccines.

The authors all support safe vaccine public policy, and recognize the historic contribution vaccines have made to public health. These findings are offered as observations of previously unpublished adverse events apparently related to the MMR vaccine, in the hope of furthering interest in safer vaccines.

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Declared potential conflicts. Drs. Bradstreet and Wakefield have, based on similar observations, been paid to prepare a report for the Legal Services Commission in the U.K. None of these cases relate directly to that litigation. Dr. Wakefield is a named inventor on viral diagnostics patents. Parents of two of the children are seeking compensation under the National Vaccine Injury Compensation Program in the U.S. The cases were filed after the detection of MV in the CSF, not prior to these findings.

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