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Abnormal Measles-Mumps-Rubella Antibodies and CNS Autoimmunity in Children with Autism

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Key Words
Autoantibodies · Autism · Autoimmunity · Measles virus · Measles-mumps-rubella antibodies · Vaccines

Abstract
Autoimmunity to the central nervous system (CNS), especially to myelin basic protein (MBP), may play a causal role in autism, a neurodevelopmental disorder. Because many autistic children harbor elevated levels of measles antibodies, we conducted a serological study of measles-mumps-rubella (MMR) and MBP autoantibodies. Using serum samples of 125 autistic children and 92 control children, antibodies were assayed by ELISA or immunoblotting methods. ELISA analysis showed a significant increase in the level of MMR antibodies in autistic children. Immunoblotting analysis revealed the presence of an unusual MMR antibody in 75 of 125 (60%) autistic sera but not in control sera. This antibody specifically detected a protein of 73–75 kD of MMR. This protein band, as analyzed with monoclonal antibodies, was immunopositive for measles hemagglutinin (HA) protein but not for measles nucleoprotein and rubella or mumps viral proteins. Thus the MMR antibody in autistic sera detected measles HA protein, which is unique to the measles subunit of the vaccine. Furthermore, over 90% of MMR antibody-positive autistic sera were also positive for MBP autoantibodies, suggesting a strong association between MMR and CNS autoimmunity in autism. Stemming from this evidence, we suggest that an inappropriate antibody response to MMR, specifically the measles component thereof, might be related to pathogenesis of autism.

Introduction

Autism is an early-onset disorder of the developing central nervous system (CNS), the etiology and pathogenesis of which is not known. The disorder causes severe deficits of higher mental functions such as social interaction, language, communication, imagination and cognition. While autism affects over one half of a million Americans and many more worldwide, very little is known about the etiology and pathogenesis of the disorder. Current theories include genetic factors, immune factors, environmental factors, neural factors and yet other unidentified factors. Owing to faulty immune regulation in autistic children [10, 12, 16, 21, 23], we focused our attention on autoimmune mechanism of pathogenesis for autism [14–17, 19, 20]. Because autoimmune diseases are generally suspected of being triggered by viruses, we recently studied virus serology in autism [15, 17]. We

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found that many children with autism had elevated levels of antibodies to measles virus (MV) but not of antibodies to human herpesvirus-6 (HHV-6), cytomegalovirus or rubella virus (RV). Moreover, the elevated level of measles antibodies was strongly associated with brain autoantibodies, which led us to postulate a pathogenetic association of MV to autoimmunity in autism [15, 17]. To further determine the source of this measles infection, we explored the possibility of an abnormal or inappropriate antibody response to MMR in relation to CNS autoimmunity. As described here, several children with autism have unusual MMR antibodies that showed a temporal association with myelin basic protein (MBP) autoantibodies that was used as a marker of CNS autoimmunity in autism.

Materials and Methods

We conducted a laboratory study of MMR antibodies and MBP autoantibodies in sera of autistic and control children. Since this study was an extension of our ongoing research, we used previously collected serum samples that were stored frozen at –20°C [14–17]. The study included a total of 217 children: 125 autistic children (aged 4–10 years) and 92 control children (58 normal children aged 5–13 years, 6 normal siblings aged 6–9 years, and 28 other disease children aged 4–12 years with behavioral disturbances other than autism). The immunization records showed that all children had their MMR immunization but none had any history of a rash or wild-type MV infection. The clinical diagnosis of autism was made essentially according to the standard DSM-IIIR criteria of the American Association of Psychiatrists, Washington, D.C., USA, as previously described [14–17]. The study included children with a firm diagnosis of autism only. The Institutional Review Board reviewed and approved our research protocol that involved the use of human serum samples only. At the time of blood sampling or a minimum of 2 weeks before the blood drawing, none of the patients or controls was taking any prescription medications such as antipsychotic or neuroleptic drugs. The MMR antibodies were detected initially by enzyme-linked immunosorbent assay (ELISA) for serum titration, but afterwards they were detected by immunoblotting assay for serum screening. In each assay method, the Measles-Mumps-Rubella (MMR)-II Vaccine (Merck, West Point, Pa., USA) was used as the antigen. Autoantibodies to brain MBP (Upstate Biotechnology, Lake Placid, N.Y., USA) were detected by immunoblotting as routinely performed in our laboratory. All immunoassays were developed in-house, simply because of the unique nature of the study and because they are presently not available from any commercial source.

The MMR antibody ELISA was adapted from our previous ELISA method [18]. Briefly, the microwells of a Costar microtiter plate (Corning, Corning, N.Y., USA) were coated with MMR antigen dissolved in PBS buffer, pH 7.4. The plate was washed three times with PBS-Tween (0.05%) buffer. 100 µl/well of PBS buffer in the blank microwells or human serum, prediluted to four dilutions (1:50, 1:100, 1:200 and 1:400), in test microwells were pipetted. The plate was incubated at room temperature for 1 h. After three washings with PBS-Tween buffer, 100 µl/well of 1:500-diluted goat anti-human-IgG-alkaline phosphatase (Sigma, St. Louis, Mo., USA) were pipetted. The plate was incubated at room temperature for 1 h. The plate was washed three times again, followed by the addition of 100 µl/well of a substrate solution (1 mg/ml of *p*-nitrophenylphosphate in 50 m*M* sodium bicarbonate buffer, pH 9.6, containing 1 m*M* magnesium chloride). The color reaction was stopped with 20 µl/well of 1 *N* NaOH and the plate was read at 405 nm using a Microplate Reader model 3550 (Bio-Rad, Richmond, Calif., USA). After blank subtraction, the absorbance readings were converted to arbitrarily defined EIA units (0.01 OD = 1 EIA unit).

Immunoblotting assay was performed essentially according to our published method [17, 20] with MMR or MBP as the screening antigen and prestained protein standards (Bio-Rad). Briefly, proteins were separated in 12% Ready Gels (Bio-Rad) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). They were transferred to nitrocellulose membranes by the double sandwich technique, followed by blocking with 1% bovine serum albumin in TBS buffer. The membranes were air-dried and stored at room temperature. For immunoassay, narrow blots (3–4 mm wide) were incubated with appropriately diluted patient or control sera for 1 h. After four washings with TBST (TBS buffer containing 0.05% Tween-20, the blots were incubated for 1 h with alkaline phosphatase conjugated goat anti-human polyvalent immunoglobulins (Sigma). After four washings with TBST, the blots were developed in substrate solution according to instructions from the manufacturer of the AP substrate kit (Bio-Rad). A reaction was scored positive only if a purplish-blue band was visualized. In some experiments, the presence of viral proteins in MMR blots was detected with monoclonal antibodies to MV hemagglutinin (HA), MV nucleoprotein (MV-NP), RV or MuV (Chemicon International, Temecula, Calif., USA), followed by immunodetection with goat anti-mouse-IgG-alkaline phosphatase; all other assay conditions were the same as aforementioned. For molecular weight determination, we simultaneously ran prestained SDS-PAGE protein standards (Bio-Rad) that included myosin (207 kD), β-galactosidase (121 kD), bovine serum albumin (81 kD), ovalbumin (51.2 kD), carbonic anhydrase (33.6 kD), soybean trypsin inhibitor (28.6 kD), lysozyme (21.1 kD), and aprotinin (7.5 kD).

Results

At first, it is important to point out that we chose to use MMR as the screening antigen, simply because it is the immunizing antigen when children are vaccinated with MMR vaccine. Therefore, the antibodies to MMR will be a true measure of seroconversion for this triple or polyvalent vaccine, instead of antibodies to measles, mumps or rubella viral proteins that are individually used for measuring virus serology in routine practice. Initially, to study the effect of serum dilution, the MMR antibody level was measured by ELISA in sera of randomly selected 24 autistic children, 14 normal children and 16 other children with conditions besides autism. As quantified by ELISA, the serum level of MMR antibodies is summarized in figure 1. Autistic children, at each of the four serum dilu-

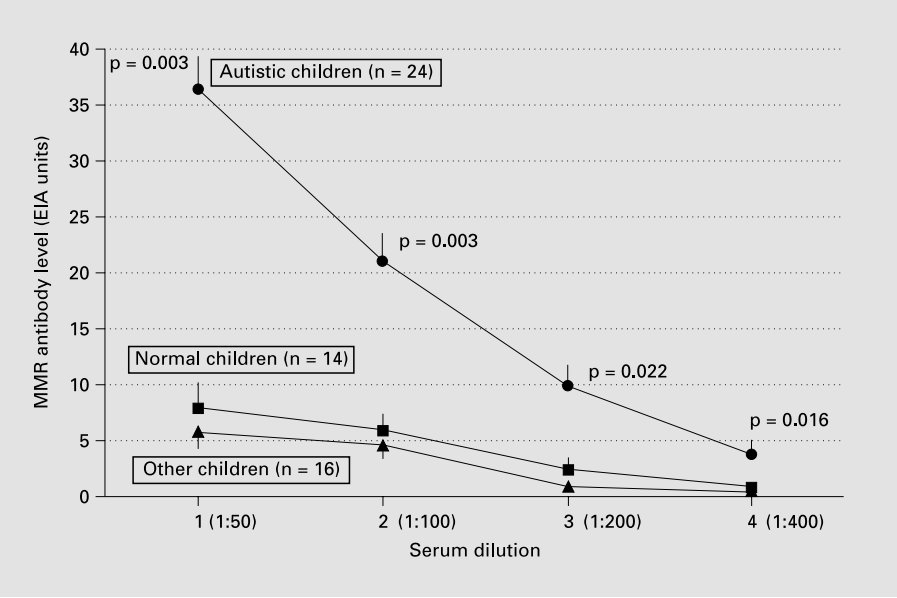

Fig. 1. ELISA detection of MMR antibodies in autism. At various serum dilutions, the MMR antibody levels are shown for autistic children (n = 24, solid circles, top line), normal children (n = 14, solid squares, middle line), and other disease children (n = 16, solid triangles, bottom line). Statistically, as evaluated by Student's t test, the MMR antibody level was significantly increased in autistic children. Data are shown as means ± SE.

tions tested, had a significantly higher level of MMR antibodies when compared to normal children or other disease children. The greatest increase (over 7-fold) was observed at 1:50 dilution of autistic sera. The ELISA method was used mainly to determine a suitable screening dilution of serum that was found to be a 1:50 dilution. Subsequently, all sera were screened at this serum dilution by the immunoblotting method because this method permits the analysis of proteins to which antibodies specifically bind to, and that was the primary objective of this study.

Immunoblotting analysis of all 217 sera revealed that 75 out of 125 autistic sera, but none of the 92 control sera, had antibodies to MMR and MBP. As shown in figure 2, autistic sera showed an immunopositive reaction to a protein band of 73–75 kD in the MMR blot (fig. 2, lane B) but control sera did not show this reaction (fig. 2, lane A); no other protein band was immunopositive in this assay. Moreover, the same protein band in MMR blots showed an immunopositive reaction to MV-HA monoclonal antibodies (fig. 3, left lanes) but not to MV-NP monoclonal antibodies (fig. 3, right lanes). The MMR blots were immunonegative for monclonal antibodies to RV or MuV (fig. 4). Consistent with previous reports [5, 15, 19, 20], autistic sera contained autoantibodies to 18.5- to 20-kD MBP (fig. 2, lane D), which is the known molecular weight of the authentic bovine brain MBP used in this study. The control sera were negative for MBP autoantibodies (fig. 2, lane C).

Based on immunoblotting data analysis, we found that 75 out of 125 (60%) autistic children were positive for MMR antibodies whereas 70 out of 125 (56%) autistic children had MBP autoantibodies (fig. 5). Neither of these two types of antibodies was detected in control children (normal children and other disease children). Furthermore, according to our immunoblotting data analysis, the autistic group showed an intriguing correlation between MMR antibody and MBP autoantibody, i.e. over 90% of the MMR antibody-positive autistic sera were also positive for MBP autoantibodies (fig. 5). This correlation was absent in the control group because the children in this group were negative for MMR antibodies as well as MBP autoantibodies.

Discussion

Several studies worldwide have suggested that immune factors such as autoimmunity may play a critical role in the pathogenesis of autism [10, 12, 14–17, 19, 20]. There is evidence for immunogenetic susceptibility factors [24] and family clustering of autoimmune diseases amongst families with autistic children [4]. Autistic children have numerous immune abnormalities: serum IgG3 increase [16], serum IgA decrease [7, 12], reduced number and function of lymphocytes, especially T helper (CD4+) cells and natural killer (NK) cells [10, 12, 21, 23], and increased plasma levels of autoimmunity-specific cyto-

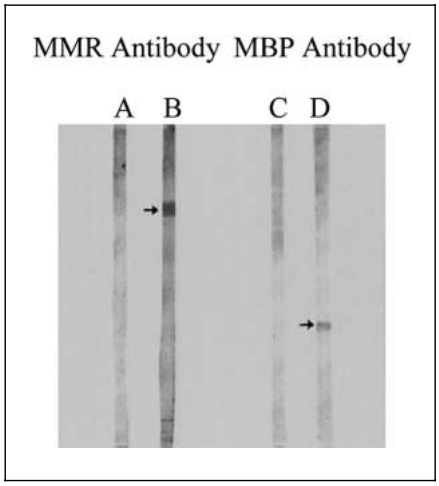

Fig. 2. Representative immunoblots of MMR antibodies and MBP autoantibodies. As described in the text, proteins in MMR and MBP blots were incubated with autistic or control serum, and probed with alkaline phosphatase-conjugated goat anti-human polyvalent immunoglobulins. Note that the autistic sera (lanes B and D), but not the control sera (lanes A and C), showed antibody-positive reactions with 73- to 75-kD protein in the MMR blot and 18.5- to 20-kD protein in the MBP blot, respectively. In 12% acrylamide gels, the MMR protein band (Rf = 17.5 mm) migrated slightly faster than the bovine serum albumin (Rf = 16.4 mm) when compared to other prestained protein standards (Cat. No. 161-0318, Bio-Rad).

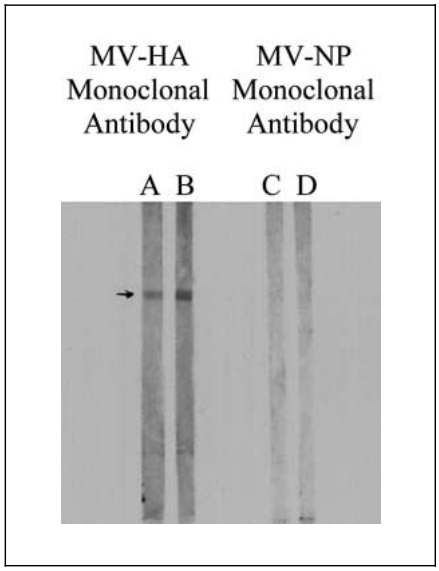

Fig. 3. Representative MMR immunoblots reacted with monoclonal antibodies to MV proteins. For this purpose, the MMR blots were separately incubated with two dilutions (1:100 and 1:50) of either MV-HA monoclonal antibodies or MV-NP monoclonal antibodies, and probed with goat anti-mouse-IgG-alkaline phosphatase. Note that the MV-HA monoclonal antibody (lanes A and B), but not the MV-NP monoclonal antibody (lanes C and D), showed an immunopositive reaction with 73- to 75-kD band of the MMR blot.

kines such as interleukin-2, interleukin-12, and interferon-γ [14]. Increased frequency of certain immunogenetic factors (C4B null allele, extended haplotype B44-SC30-DR4 and hypervariable HLA-DRb1 region) has also been shown in some children with autism [24]. Many autistic children have organ-specific autoantibodies, in particular autoantibodies to brain myelin-derived MBP [15, 17, 19, 20]. Furthermore, a considerable number of autistic children show significant improvements of autistic characteristics when treated with immune therapy such as oral autoantigen [15], intravenous immunoglobulin [7] or transfer factor [5]. Collectively, these immune abnormalities and/or immune therapies are consistent with an autoimmune basis of pathogenesis in autism.

Viruses are commonly associated with autoimmune diseases, albeit the paucity of experimental evidence. The trigger mechanism for autoimmunity in autism is not known but viral associations have been described [2, 8]. Autistic children harbor significantly higher than normal levels of measles antibodies but not of HHV-6, rubella or cytomegalovirus antibodies [15, 17]. The specific increase of measles antibody level was also consistent with a serological association between MV and autoimmunity in autism, which led us to postulate an etiological link of MV with autism [15, 17]. As reported here, a significant increase of MMR antibody level was found in autistic children. Moreover, the MMR antibody showed immunopositive reaction to a 73- to 75-kD protein of MMR in 60% of autistic children in the study. This was an important finding because the molecular weight of MMR protein that reacted positive for MMR antibodies resembled the molecular weight of a measles protein, known as HA antigen. Indeed, the MMR band contained MV-HA antigen as it was immunopositive for monoclonal antibodies to MV-HA, but not for monoclonal antibodies to MV-NP. In preliminary data not included here, we recently found that the MV-HA monoclonal antibody but not the MV-NP monoclonal antibody almost completely blocked the binding of MMR antibody (antibody-positive autistic sera) to the MMR protein band on immunoblots. Therefore, these indirect studies suggest that MMR antibodies in autistic sera are most likely directed towards HA antigen of MV. Moreover, the 73 to 75-kD band of MMR did not contain RV or MuV as this band was immunonegative for monoclonal antibodies to either of these two viruses. Relative to autistic children, the control children

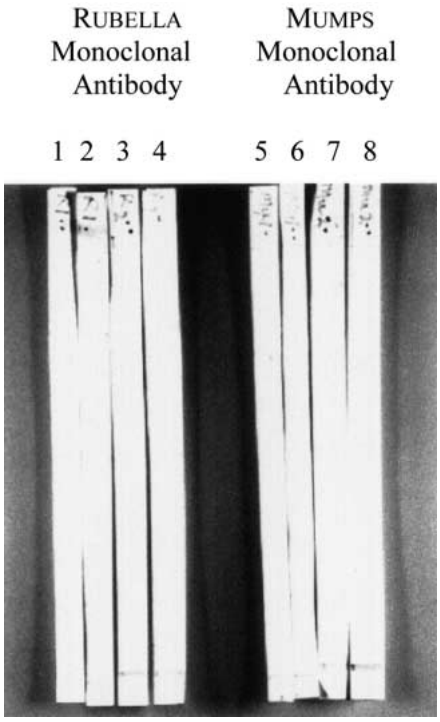

Fig. 4. Representative MMR immunoblots reacted with monoclonal antibodies to RV or MuV. The MMR blots from 4 separate SDS-PAGE runs were incubated with monoclonal antibodies (1:100 dilution) to RV or MuV, and probed with goat anti-mouse-IgG-alkaline phosphatase. Note that the MMR blots were negative in these immunoassays.

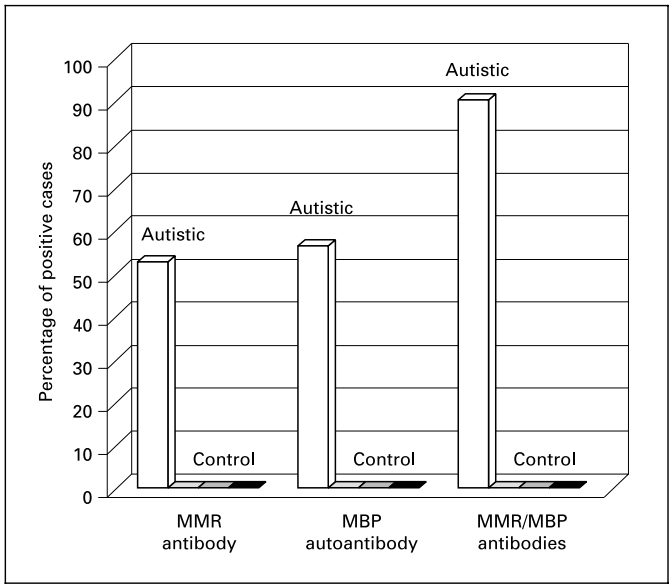

Fig. 5. Distribution of MMR and MBP antibodies in autistic and control children. After antibody screening by immunoblotting, the percentage of antibody-positive sera was calculated in each study group. This was plotted against the antigen source assayed. Note that only the autistic group showed positive reactions (vertical bars) but the control group that included normal children (baseline box 1), normal siblings (baseline box 2), and other disease children (baseline box 3) was negative.

had low levels of MMR antibodies that were immunonegative for MMR-derived MV-HA antigen. Thus it seems plausible that autistic children elicited an inappropriate or abnormal antibody response to MMR that was directed against the MV-HA antigen. Undoubtedly, more research is necessary on this topic but we are tempted to speculate that a faulty immunoregulation or immunogenetic factors may determine why only autistic children produce these abnormal antibodies to MMR-derived protein (73–75 kD) that appears to be the HA antigen of MV. Alternatively, the difference between the autistic and control children may be due to a structural modification (or mutation) of antigenic determinant that is recognized by MMR antibodies.

Immunization with vaccines is the best preventive measure against deadly infections available to mankind today. Because vaccines are given to healthy subjects, almost exclusively to children, the safety of vaccines must be as absolute as humanely possible. Although the risk-to-benefit equation strongly favors vaccination, there are some serious side effects, albeit extremely rare, that deserve scientific attention. For instance, aseptic meningitis [6] and cerebellar ataxia [11] have been described in children immunized with MMR. However, the basis of how vaccines react adversely in some cases remains virtually unknown. It is quite possible that vaccines in a small population of genetically predisposed children may react inappropriately, simply because of their immature immune system or some other unknown risk factors such as immunodeficiencies, allergies, chemical toxins or chronic psychological stress [3]. However, none of these factors has so far been investigated in autism.

In recent years, the immunization-autoimmunity topic has gained quite a bit of public attention. This is quite possibly because autoimmune diseases are the commonest manifestations of immunizations [1, 13]. The MMR has been insinuated as a culprit of gastrointestinal problems in some children with autistic characteristics [22]. Approximately one half of the parents with autistic children reported autistic regression after the MMR immunization [17]. Moreover, a serological association of MV

with autoimmunity was found in autistic children who did not have a wild type measles infection but they did have the MMR immunization [17]. And, as described herein, autistic children showed a serological correlation between MMR and brain autoimmunity, i.e., over 90% of MMR antibody-positive autistic sera also had autoantibodies to brain MBP. This is quite an intriguing observation in favor of a connection between atypical measles infection and autism; an atypical infection usually refers to infection that occurs in the absence of a rash. An atypical measles infection in the absence of a rash and unusual neurological symptoms was recently described to suggest the existence of a variant MV in children and adults [9]. In light of these new findings, we suggest that a considerable proportion of autistic cases may result from an atypical measles infection that does not produce a rash but causes neurological symptoms in some children. The source of this virus could be a variant MV or it could be the MMR vaccine. Scientifically, therefore, it is instructive to consider both these possibilities and uncover them through experimental research. We think that this is an extremely important public health issue, quite simply because some scientists have recently warned us about the emergence of a mutant MV that causes fatal illnesses in man [9]. If this is the case, then new vaccination strategies will be required to combat mutant measles infection. While more research is necessary to establish a pathognomonic role for MMR/MV, we are currently exploring the role of virus-induced autoimmunity and our future research is aimed at characterizing the molecular basis of cellular and humoral immunity to viral antigens in children with autism.

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