# BRIEF COMMUNICATION

## Antibodies to Myelin Basic Protein in Children with Autistic Behavior

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Based on a possible pathological relationship of autoimmunity to autism, antibodies reactive with myelin basic protein (anti-MBP) were investigated in the sera of autistic children. Using a screening serum dilution of 1:400 in the protein-immunoblotting technique, approximately 58% (19 of 33) sera of autistic children ($\leq 10$ years of age) were found to be positive for anti-MBP. This result in autistics was significantly ($p \leq .0001$) different from the controls (8 of 88 or only 9% positive), which included age-matched children with normal health, idiopathic mental retardation (MR) and Down syndrome (DS), and normal adults of 20 to 40 years of age. Since autism is a syndrome of unknown etiology, it is possible that anti-MBP antibodies are associated with the development of autistic behavior. © 1993 Academic Press, Inc.

## INTRODUCTION

The behavioral syndrome of autism in children is generally considered to be a pervasive neuro-developmental disorder identified by neuropsychiatric manifestations that include few or no language and imaginative skills, repetitive-rocking and self-injurious behavior, and abnormal responses to sensations, people, events, and objects. The cause of the syndrome is not known but it may involve one or more factors such as environmental factors (perhaps a virus infection), genetic factors, immunological factors, and yet undiscovered neuropathological and biochemical factors.

An immune hypothesis involving autoimmunity as one possible mechanism of pathogenesis in autism has recently been suggested (Weizman, Weizman, Szekely, Wijsenbeek, & Livni, 1982; Todd & Ciaranello, 1985; Stubbs, 1987; Singh, Fudenberg, Emerson, & Coleman, 1988). Supporting evidence for this hypothesis is largely based on a family study of infantile autism in the presence of autoimmune disease (Money, Borrow, & Clarke, 1971), abnormalities of both cellular and humoral immunity in about one-half of autistic children (Stubbs, Crawford, Burger, & Vandenbark, 1977; Weizman et al., 1982; Todd & Ciaranello, 1985; Warren, Foster, Margaretten, & Pace, 1986; Singh et al., 1988; Warren, Yonk, Burger, Cole, Odell, Warren, White, & Singh, 1990; Yonk, Warren, Burger, Cole, Odell, Warren, White, & Singh, 1990), and altered levels of soluble antigens of immunocyte activation (Singh, Warren, Odell, & Cole, 1991). In the present study, we investigated the presence of antibodies reactive to myelin basic protein (anti-MBP) in the sera of children with autism.

## MATERIALS AND METHODS

*Subject population.* In this study, 33 autistic children ($\leq 10$ years of age) were studied as compared to controls which included 18 normal children ($\leq 10$ years of

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age), 20 children with idiopathic mental retardation (≤10 years of age), 12 children with Down's syndrome (≤10 years of age), and 38 normal adults in the age range of 20 to 40 years. The diagnosis of autism was made according to the recently established criteria as outlined in the Diagnostic and Statistical Manual of Mental Disorders, Third revised edition (DSM-III-R), American Psychiatric Association, Washington, D.C. As previously described (Warren et al., 1990; Singh et al., 1991), the syndrome of autism was identified by at least one pediatric neuropsychiatrist and confirmed by a clinical child psychologist. The children with idiopathic mental retardation (MR) showing an Intelligence Quotient (IQ) ≤70 and adaptive behavior deficits or Down's syndrome were referred to us by parents and school psychologists, and they were included as a disease-control group since approximately 60% of autistic children have IQ of 70 or lower. Five milliliters of venous blood was drawn from each donor by the standard method of venipuncture after parental consent was obtained on behalf of each child. At the time of blood drawing, none of the autistic or retarded children was taking any prescription medication or antipsychotics. After the blood samples clotted, they were centrifuged at 1500 rpm for 10 min and the serum was collected for storage at −20°C.

Detection of antibodies to myelin basic protein (anti-MBP). Antibody binding to MBP was detected by Western-immunoblotting technique using rabbit MBP (18.5 kDa) as the screening antigen purchased from Calbiochem (La Jolla, CA). Using a Mini-Gel apparatus (Hoffler Scientific), MBP was separated in 10% gels by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol according to the method of Laemmli (1970). The gels were run for about 2 h at approximately 80 V, and the protein bands were detected by staining with Coomassie blue. The proteins from the unstained gels were blotted onto the nitrocellulose membrane by electrotransfer for 16 to 18 h at 25 V (Towbin, Staehelin, & Gordon, 1979). The blots were cut into narrow strips of 8 mm width and used for immunodetection. The blots were soaked while slowly shaking in the blocking buffer (5% nonfat dry milk in TBS buffer which contained 0.02 $M$ Tris–HCl, pH 7.5, 0.5 $M$ NaCl, and 0.05% Tween-20) for about 90 min at room temperature (22°C). The blots were washed four times of 5 min with each treatment of TBS buffer and incubated with primary antibody which was contained in the patient or control sera (prediluted to a screening dilution of 1:400) for 90 min at room temperature, followed by four washings as mentioned above. The blots were reacted with a secondary antibody (goat-antihuman-IgG-alkaline phosphatase purchased from Bio-Rad, Richmond, CA) prediluted to a 1:2000 dilution in Tris-buffer containing 0.01 $M$ Tris–HCl, pH 8.0, 0.05 $M$ NaCl, 1 m$M$ MgCl$_2$, and 1% bovine serum albumin. After incubating 90 min at room temperature, the blots were washed four times, and developed within 10 min in the alkaline phosphatase (AP) substrate reagent solution prepared according to the kit manufacturer's recommendation (Bio-Rad). A reaction was scored positive whenever a purplish-blue protein band was detected. In some experiments, the primary antibody was a mouse monoclonal antibody (1:1000 dilution) to MBP which was detected by goat–anti-mouse-IgG-alkaline phosphatase (1:1500 dilution), and this represented a positive test of anti-MBP detection. The monoclonal antibody, which reacts with residues 130–137 of the MBP of myelin from human, bovine, monkey, and rat brains, was purchased from Boehringer-Mannheim (Indianapolis, IN). Rabbit MBP antigen was replaced in two experiments by human MBP which was kindly provided by Professor Naren L. Banik (Department of Neurol-

ogy, Medical University of South Carolina, Charleston, SC). All sera were coded and tested in a double-blind fashion.

## RESULTS AND DISCUSSION

After SDS–polyacrylamide gel electrophoresis, the Coomassie blue staining of rabbit MBP showed a single protein band (Fig. 1). Typical examples of a positive reaction of anti-MBP by protein-immunoblotting technique are shown in Fig. 2. The blot in lane 1 is the reaction of monoclonal antibody to rabbit MBP, lanes 2 through 5 are reactions with sera of autistic children, and lane 6 is no reaction with the serum of a normal child. All sera were screened at a dilution of 1:400, which was chosen after titration to reduce the nonspecific background. As summarized in Table 1, the anti-MBP positive reaction was found in 19 of 33 (58%) sera from autistic children. In contrast, only 8 of 88 (9%) control sera were positive. Among these control sera, 3 of 20 (15%) sera from MR children, 4 of 18 (22%) sera from normal children, only one of 38 sera from normal adults (25 to 40 years of age) but none of the 12 sera from DS children showed this antibody positive reaction. For the purpose of cross-reactivity, six positive sera from autistic children and six negative sera from healthy children were tested in two experiments using human MBP as the antigen. The result of this exercise was the same as that found with rabbit MBP, suggesting cross-reactivity of the antibodies despite the fact that not all sera could be screened due to extremely limited amounts of human MBP available to us. The statistical analysis of the data revealed a significant ($p \leq$ .0001) difference between the autistic group and the control group.

It is not clear why sera from 4 of 18 (22%) normal children and one normal adult were also positive for anti-MBP. This result however is not unusual since a small proportion of normal population is known to have serum antibodies to central nervous system antigens (anti-CNS). For instance, up to 21% of normal children (which closely resembles our result of 22%) without any neurologic manifestations were recently found to be positive for anti-CNS by Western-immunoblotting technique (Plioplys, Greaves, & Yoshida, 1989). The results of our study also sug-
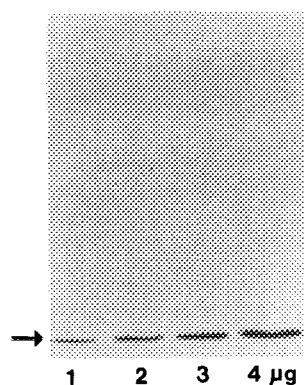


Fig. 1. SDS–PAGE analysis of rabbit myelin basic protein. As shown, different amounts of rabbit MBP (1, 2, 3, and 4 μg) were subjected to SDS–PAGE analysis according to the details given under Materials and Methods. The proteins were stained for about 4 h at room temperature with 0.125% (w/v) of Commassie brilliant blue R-250 dye prepared in 40% (v/v) methanol and 10% (v/v) glacial acetic acid, and destained in the large excess of the solvent containing 5% (v/v) methanol and 7.5% (v/v) of glacial acetic acid.
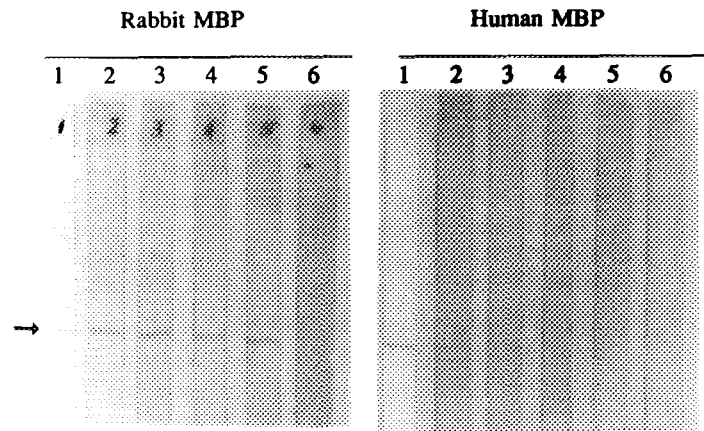
Rabbit MBP          Human MBP



FIG. 2. Typical illustrations of anti-MBP immunoblots. Approximately, 4 μg of rabbit or human MBP was separated by SDS–PAGE followed by electrotransfer and immunodetection as described under Materials and Methods. A positive reaction was detected for monoclonal antibody to MBP used as a positive control (lane 1) and for four sera from autistic children (lanes 2 to 5) whereas a negative reaction was shown by a normal child serum (lane 6). All sera were screened at a predetermined 1:400 dilution.

gested that the humoral immune response to MBP may be related in some way to mental retardation since approximately 60% of autistic children had an IQ of 70 or lower. However, this likelihood is very remote since the detectable incidence of anti-MBP in age-matched MR children was virtually negligible (none of the 12 DS children and only 3 of 20 children with idiopathic MR were positive). It should also be mentioned that seizure activity or antipsychotic drugs are probably not related to the production of anti-MBP since there was neither the history of seizures nor the intake of antipsychotics (at least not at the time of blood drawing) among autistic or retarded children that we have studied thus far.

Immunological studies of autistic patients have revealed certain features that are also found in patients with other autoimmune diseases. There is a genetic predisposition for several autoimmune diseases (Shoenfeld & Isenberg, 1989) like Grave's thyroid disease, rheumatoid arthritis, and insulin-dependent diabetes, and likewise, autism shows a greater concordance rate in monozygotic twins than

TABLE 1
Summary of Anti-MBP Screening by Immunoblotting Technique

| Blood donors (Age) | Total number of sera tested | Number of sera positive | Percentage positive |
|---|---|---|---|
| Children with autism (≤10 years) | 33 | 19* | 57.6% |
| Total controls | 88 | 8 | 9% |
| (a) Children with MR (≤10 years) | 20 | 3 | 15% |
| (b) Children with DS (<10 years) | 12 | 0 | 0% |
| (c) Normal children (≤10 years) | 18 | 4 | 22% |
| (d) Healthy adults (20–40 years) | 38 | 1 | 2.6% |

* This result in autistics ($n = 33$) was significantly ($p \leq .0001$) different when compared with controls ($n = 88$) collapsed together for the purpose of $\chi^2$-statistical analysis with 2 × 2 contingency tables (contingency coefficient was 0.46).

in the normal population (Folstein & Rutter, 1988). Autism is four to five times more prevalent in boys than in girls—a gender factor which is also common in systemic lupus erythematosus (SLE), Grave's disease, and ankylosing spondylitis. Our group recently described that autism, like other autoimmune diseases, also displays associations with certain alleles of the major histocompatibility (MHC) complex located on chromosome 6 (Warren, Singh, Cole, Odell, Pingree, Warren, & White, 1991; Warren, Singh, Cole, Odell, Pingree, Warren, DeWitt, & McCullough, 1992). Moreover, triggering by microorganisms is thought to be an important feature of autoimmune diseases; whether a similar event is associated with autism is not known but there are coincidental findings of congenital rubella (Chess, 1971) and cytomegalovirus (Stubbs, 1987; Ivarsson et al., 1990) indicating prior exposure to these microbial agents. Recently, we also found that certain soluble antigens of immunocyte activation are elevated in the sera of autistic children (Singh et al., 1991), which is in concordance with similar findings in other autoimmune diseases like SLE (Huang, Perrin, Miescher, & Zubler, 1988) and multiple sclerosis (Trotter, Clifford, Anderson, van der Veen, Hicks, & Banks, 1988).

Based on aforementioned parallels between autism and other autoimmune diseases, it is possible that autoimmunity may explain a subset of autism. Inflammation or lymphocyte infiltrate has not been found or studied in the brain of autistic children, but several autistic children display abnormalities of immune components including autoantibodies to brain antigens. As reported independently by three groups of researchers (Singh et al., 1988; Stubbs et al., 1977; Warren et al., 1986), the lymphocyte proliferation by phytohemagglutinin, concanavalin A, and pokeweed mitogens are generally depressed in 40 to 50% of autistic patients. Recently, our group also reported that the blood proportions of $CD4^+$ T helper cells and a suppresor–inducer ($CD4CD45RA^+$) subset thereof are significantly depressed in autistic patients (Warren et al., 1990; Yonk et al., 1990). Although the dot blot analysis of antibrain antibody titers in infantile autism did not support a generalized, ongoing, antibody-mediated immune response, it was suggested that only a few brain antigens might be involved in the development of autistic symptoms (Todd, Hickok, Anderson, & Cohen, 1988). Some indication of this possibility may be rendered from studies which showed blocking by autistic patient sera of [$^3$H]serotonin binding to brain tissue homogenates presumably due to autoantibodies to putative serotonin receptors (Todd & Ciaranello, 1985), serum antibodies to neuron–axon filament proteins (Singh et al., 1988), and anti-MBP in approximately 58% of autistic children (Table 1). Similar to the work of Todd and Ciaranello (1985), our unpublished research has also found evidence of blocking autoantibodies to the brain serotonin receptors in the sera of autistic children.

At present, a cause or an effect relationship between antibodies to MBP and autism cannot be defined very well. It is possible that these antibodies are epiphenomena and not related to autism. However, since autistic children are known to display quite varied behavioral manifestations, more than one factor may be involved in the causation of the syndrome. Consequently, we hypothesize that the development of humoral immune response to MBP should be regarded as the proponent of immunopathogenesis in a subset of autism. At birth, there is very little myelin in the brain, and myelination may not be complete until age 10 years or older in the normal child (Trevarthen, 1974). This is also the age-group of

autistic children that we studied for antibodies to MBP (Table 1). Moreover, delayed or incomplete myelination in the corpus callosum (which is the largest myelinated area of the brain) has been suggested as the basis of auditory processing problems in some children with learning disabilities (LD) (Musiek, Gollegly, & Baran, 1984). In light of this knowledge, if an immunological assault perhaps secondary to a virus infection were to occur prenatally or postnatally during infancy or early childhood, it could possibly result into poor myelination or abnormal function of the neuron–axon myelin. The latter may be a critical factor in the development of neurobehavioral problems in some cases of the syndrome, and should be worthy of future research for the understanding of a pathological basis of autism.

## ACKNOWLEDGMENTS

## REFERENCES

Chess, S. (1971). Autism in children with congenital rubella. *J. Aut. Childh. Schizophr.* 1, 33–47.

Folstein, S., & Rutter, M. (1988). Autism: Familial aggregation and genetic implications. *J. Aut. Develop. Disord.* 18, 3–30.

Huang, Y. P., Perrin, L. H., Miescher, P. A., & Zubler, R. H. (1988). Correlation of T and B cell activities in vitro and serum IL-2 levels in systemic lupus erythematosus. *J. Immunol.* 141, 827–833.

Ivarsson, S. A., Bjerre, I., Vegfors, P., & Ahlfors, K. (1990). Autism as one of several abnormalities in two children with congenital cytomegalovirus infection. *Neuropediatrics* 21, 102–103.

Laemmli, E. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.

Money, J., Bobrow, N. A., & Clarke, F. C. (1971). Autism and autoimmune disease: A family study. *J. Aut. Childh. Schizophr.* 1, 146–160.

Musiek, F. E., Gollegly, K. M., & Baran, J. A. (1984). Myelination of the corpus callosum and auditory processing problems in children: theoretical and clinical correlates. *Semin. Hearing* 5, 231–241.

Plioplys, A. V., Greaves, A., & Yoshida, W. (1989). Anti-CNS antibodies in childhood neurologic diseases. *Neuropediatrics* 20, 93–102.

Shoenfeld, Y., & Isenberg, D. (1989). The genetic components in autoimmunity. In *The Mosaic of Autoimmunity*, Chap. 5, pp. 169–228. Elsevier: Amsterdam.

Singh, V. K., Fudenberg, H. H., Emerson, D., & Coleman, M. (1988). Immunodiagnosis and immunotherapy in autistic children. *Ann. N.Y. Acad. Sci.* 540, 602–604.

Singh, V. K., Warren, R. P., Odell, J. D., & Cole, P. (1991). Changes of soluble interleukin-2, interleukin-2 receptor, T8 antigen, and interleukin-1 in the serum of autistic children. *Clin. Immunol. Immunopath.* 61, 448–455.

Stubbs, E. G. (1987). Does intrauterine cytomegalovirus plus autoantibodies contribute to autism? In L. Wing (Ed.), *Aspects of autism: Biological research*, Gaskell Psychiatry Series, pp. 91–101.

Stubbs, E. G., Crawford, M. L., Burger, D. R., & Vandenbark, A. A. (1977). Depressed lymphocyte responsiveness in autistic children. *J. Aut. Childh. Schizophr.* 7, 49–55.

Todd, R. D., & Ciaranello, R. D. (1985). Demonstration of inter- and intraspecies differences in serotonin binding sites by antibodies from an autistic child. *Proc. Natl. Acad. Sci. USA* 82, 612–616.

Todd, R. D., Hickok, J. M., Anderson, G. M., & Cohen, D. J. (1988). Antibrain antibodies in infantile autism. *Biol. Psychiat.* 23, 644–647.

Towbin, H., Staehelin, T., & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76, 4350–4354.

Trevarthen, C. (1974). Cerebral embryology and the split brain. In M. Kinsbourne & W. Smith (Eds.), *Hemispheric disconnection and cerebral function*, pp. 208–236. Charles C. Thomas: Springfield, IL.

Trotter, J. L., Clifford, D. B., Anderson, C. B., van der Veen, R. C., Hicks, B. C., & Banks, G. (1988). Elevated serum interleukin-2 levels in chronic progressive multiple sclerosis. *N. Engl. J. Med.* 318, 1206.

Warren, R. P., Foster, A., Margaretten, N. C., & Pace, N. C. (1986). Immune abnormalities in patients with autism. *J. Aut. Dev. Disord.* 16, 189–197.

Warren, R. P., Singh, V. K., Cole, P., Odell, J. D., Pingree, C. B., Warren, W. L., & White, E. (1991). Increased frequency of the null allele at the complement C4b locus in autism. *Clin. Exp. Immunol.* 83, 438–440.

Warren, R. P., Singh, V. K., Cole, P., Odell, J. D., Pingree, C. B., Warren, L. W., DeWitt, C. W., & McCullough, M. (1992). Possible association of the extended MHC haplotype B44-SC30-DR4 with autism. *Immunogenetics* 36, 203–207.

Warren, R. P., Yonk, L. J., Burger, R. A., Cole, P., Odell, J. D., Warren, W. L., White, E., & Singh, V. K. (1990). Deficiency of suppressor-inducer (CD4 + CD45RA +) T cells in autism. *Immunol. Invest.* 19, 245–251.

Weizman, A., Weizman, R., Szekely, G. A., Wijsenbeek, H., & Livni, E. (1982). Abnormal immune response to brain tissue antigen in the syndrome of autism. *Am. J. Psychiat.* 7, 1462–1465.

Williams, R. S., Hauser, S. L., Purpura, D. P., Delong, G. R., & Swisher, C. N. (1980). Autism and mental retardation. *Arch. Neurol.* 37, 749–753.

Yonk, L. J., Warren, R. P., Burger, R. A., Cole, P., Odell, J. D., Warren, W. L., White, E., & Singh, V. K. (1990). CD4 + helper T cell depression in autism. *Immunol. Lett.* 25, 341–346.