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STUDY OF CIRCULATING ANTIBODIES TO HEAT-SHOCK

PROTEINS 60 AND 70 IN AUTISTIC SUBJECTS

by

Fang-Yi Chiu

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Biology

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

1994

ACKNOWLEDGMENTS

I would like to express gratitude to my advisor, Dr. Reed Warren, for his encouragement, patience, and assistance. I am particularly grateful for his valuable assistance in the development of computer graphics. His able direction and financial support made this work possible.

Much praise and appreciation go to Dr. Bill Barnett, who gave me many suggestions on the experimental techniques. He also kindly spent his time helping me resolve problems I had while doing my experiments.

My sincere thanks to Dr. Dennis Odell for serving on my committee and introducing me to the autistic syndrome.

Special thanks to the members in the Biomedical Laboratory of the Center for Persons with Disabilities (CPD), Alma, Linda, Luise, Peggy, Roger, and Wayne. They not only shared their knowledge with me but also showed me the American culture. I am grateful.

I am deeply indebted to my parents, Chiu Chin-Chow and Shu Kwei-Mei, for their continual encouragement and financial support. Finally, my appreciation is also extended to my boyfriend, Chen Feng-Wen, for his understanding and patience.

Fang-Yi Chiu

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ABSTRACT

Study of Circulating Antibodies to Heat-Shock

Proteins 60 and 70 in Autistic Subjects

by

Fang-Yi Chiu, Master of Science

Utah State University, 1994

Major Professor: Dr. Reed P. Warren
Department: Biology

Autism is a behavioral syndrome characterized by a severe impairment of reciprocal social relations, and of verbal and nonverbal communications. Many different etiologic factors such as viral infection and genetic predisposition have been proposed to explain the development of this disorder. Immune abnormalities, such as a decreased lymphoblastic response to T-cell mitogen, defective antibody responses to rubella vaccine, and decreased numbers of T lymphocytes, also have been identified in a subpopulation of patients with autism, which implies that the development of autism in some cases may be due to autoimmune mechanisms.

Recent evidence suggests that immune response to the heat-shock proteins 60 and 70 is associated with several autoimmune diseases, including juvenile arthritis, type I diabetes, and multiple sclerosis. Therefore, in this study, the plasmas of patients with autism were examined by enzyme-

linked immunosorbent assay (ELISA) for antibodies to the heat-shock proteins 60 and 70.

The autistic subjects were found to have increased levels of antibodies against heat-shock protein 70 as compared to that of age-matched controls ($p=0.0148$). However, levels of antibodies to heat shock protein 60 in the autistic subjects showed considerable individual variation and no significant difference was found.

Abnormal immune reactions to myelin basic protein have also been found in autistic subjects. Since epitopes on myelin basic protein have been shown to crossreact with determinants on heat-shock protein 60, the similarity between anti-myelin basic protein monoclonal antibodies and anti-heat-shock protein 60 antibodies in the autistic subjects was also studied. The results showed no crossreactivity between these two antibodies.

In conclusion, the data from the study of antibodies against heat-shock protein 70 suggest an elevated immune response to heat-shock protein 70 in autistic subjects. This result implies that autism could be an autoimmune disease.

(71 pages)

CHAPTER I

INTRODUCTION

Autism is a biologically based developmental disorder arising during the first 36 months of life, which is commonly regarded as a pervasive neurodevelopmental disorder. This syndrome is identified by neuropsychiatric manifestations that include impairment of verbal or nonverbal communicative skills, bizarre responses to people's touch and objects, abnormal motoric mannerisms such as repetitive-rocking and self-injurious behavior, and ease of irritation by minor changes in the immediate environment (Ritvo and Freeman, 1978). Autism arises in all races and social classes without special distribution, and occurs three to four times more often in males than in females (Ornitz and Ritvo, 1976). Genetic, immunological, viral, and environmental mechanisms have been proposed as etiological factors for this severe behavioral disorder. However, no specific causes have been identified. Some investigations suggest that this disorder shares features with autoimmune disorders, including cell-mediated immunity and/or antibodies against brain associated antigens (e.g., myelin basic protein) (Stubbs et al., 1977; Weizman et al., 1982; Warren et al., 1986; Warren et al., 1990a; Yonk et al., 1990; Singh et al., 1993).

There is increasing evidence that heat-shock proteins (HSP) play an important role in autoimmunity, since increased antibody levels to HSP cognates have been observed in

autoimmune diseases, including rheumatoid arthritis, ankylosing spondylitis, and systemic lupus erythematosus (Bahr et al., 1988; Tsoulfa et al., 1989). HSPs belong to a large family of proteins that are highly conserved in all organisms. They are synthesized by many kinds of cells in response to a broad range of stresses (e.g., heat and heavy metals) and function to protect cells against further stress.

The extreme conservation of HSPs is a possible reason for their involvement in certain autoimmune diseases. Formation of autoantibodies may be caused by molecular mimicry (epitope homology) between microbial antigens and host proteins (Hedstrom et al., 1987). Moreover, some of the HSPs appear constitutively only after an encounter with various types of stresses. The immune system recognizes these stress-related HSPs as foreign, which results in a primary autoimmune reaction against such HSPs.

HSPs are classified into families based on their molecular sizes (e.g., 90kDa, 70kDa, 60kDa, 20kDa). Among these, the HSP70 is one of the most abundant (Laemmli, 1970; Lindquist, 1986). The HSP70 gene is localized within the major histocompatibility complex (MHC) (Sargent et al., 1989), which is of special interest, since Warren et al. (1991) have found an association between MHC genes and autism, including the extended haplotype (B44-SC30-DR4)

(Warren et al., 1992). The HSP70 gene is located within the extended haplotype and as such may be a susceptibility factor for the development of the neurobehavioral symptoms in autism.

Another important heat-shock protein is the HSP60. This protein has been associated with autoimmunity because it has a high degree of homology with HSP65 found in microorganisms. It has been reported that the level of antibodies against HSP60 in schizophrenia patients is higher than in normal controls (Kilidireas et al., 1992).

As mentioned above, studies (Weizman et al., 1982; Singh et al., 1993) have shown abnormal immune reactions against myelin basic protein (MBP) in autism. Since myelin basic protein is known to share determinants with HSP60, it seems worthwhile to investigate whether the HSP60 antibody from the autistic subjects can compete with anti-MBP monoclonal antibody for binding to the HSP60 antigen. If the antibodies do compete, one can speculate that antibody against HSP60 crossreacts with MBP in the brain, resulting in the manifestation of autism.

CHAPTER II

LITERATURE REVIEW

Autism

Autism is a rare developmental disorder with reported frequencies varying from 0.7 to 4.5 per 10,000 (Ritvo and Freeman, 1978). It is believed that the onset of this disorder occurs before birth or shortly after birth. However, autism is currently detectable beginning at about three years of age. Earlier diagnosis is difficult because there are no specific physical signs indicating autism during early infancy. Approximately 67 to 88 percent of classical autistic patients are mentally retarded (IQ < 70)(Gillberg et al., 1991). The life span of autistic subjects is the same as normal individuals. The current Diagnostic and Statistical Manual of Mental Disorders (DSM, American Psychiatric Association, 1987) defines autism as a developmental disorder demonstrating qualitative impairment in social interaction, verbal and nonverbal communications, and in imaginative activity. The most common behavioral symptoms of autistic children can be classified as the following categories:

1. Impairment of perception and relating. Disturbance of perception is common to all autistic patients. In many instances they do not responsd to either verbal commands or sounds, although sometimes they can respond to stimuli with

exaggerated reactions. These phenomena suggest that abnormal perceptions may be due to an underlying neuropatho-physiological process (Tanguay, 1976). The result of impaired perception may cause disturbed relatedness (Hermelin and O'Connor, 1970), which includes absent social smile, apparent dislike of physical contact, and no interest in playing games with others.

2. Disturbed motility. The disturbance of motility seen in autism is repeated body movements such as hand-flicking and/or twisting (APA, 1987).

3. Impairment of speech and language. Autistic children have extreme difficulty in developing language and communication skills. Almost half of the population of autistic children never acquire functional language ability (Paul, 1987).

Some other symptoms that are not among the diagnostic criteria of DSM-III-R but are common in autistic children include an abnormal response to sound (i.e., no response to a sudden explosion but irritated by ordinary noise levels), bizarre response to pain, heat, or cold (i.e., no reaction to burn), difficulty in recognizing the things they see.

To date, the etiology and pathogenesis of autism is unknown. It is believed that the behavioral syndrome of autism represents the final expression of a variety of etiological factors. These possible factors include genetic susceptibility (Folstein and Rutter, 1977), viral infection

(Chess, 1971; Stubbs, 1987), and immunological dysfunctions. The latter includes decreased responsiveness of lymphocytes to mitogens (Stubbs et al., 1977), reduced function of natural killer cells (Warren et al., 1987), reduced numbers of T helper cells (Yonk et al., 1990), deficiency of suppressor-inducer (CD4+CD45RA+) T cells (Warren et al., 1990b), an increased frequency of the null allele at the complement C4B locus (Warren et al., 1990), an increased incidence of the extended haplotype [B44-SC30-DR4], and immune abnormalities of cell-mediated immunity and/or antibodies against myelin basic protein.

Genetic factors in autism

Genetic disease is currently believed to be a possible cause of 10 to 20% of all cases of autism (Bolton and Rutter, 1990; Gillberg, 1992). In England, Folstein and Rutter (1977) reported a 36% autism concordance rate in monozygotic twins and a 0% concordance rate in same-sex dizygotic twins. They concluded that genetic factors may play a part in the etiology of autism. DeMyer et al. (1981) surveyed a large population and found that between 2% and 6% of autistic probands have autistic siblings. Ritvo et al. (1989) did an epidemiological survey of autism in Utah and found that siblings of autistic patients have a 215 times greater chance of being autistic than siblings of non-autistic individuals. The frequency of developing autism for each sibling who is born after an autistic child is 8.6%. Thus, the above

results indicate that there may be a subtype of autism that is genetically influenced.

Several types of genetic patterns have been observed in patients with autism. First, aberrations of whole or pieces of chromosomes have been associated with autism. Gillberg et al. (1991) reported that a marker chromosome (extra material from the long arm of chromosome 15) is associated with a specific autistic syndrome. In addition, a fragile site (Xq27.3) has been found in a number of autistic patients (Cohen et al., 1991).

The results of a study on familial aggregation and genetic implications by Folstein and Rutter in 1988 suggested that it is not autism itself that is inherited but rather some genetic aberrations, such as language and/or sociability abnormalities, which combine with an environmental insult such as birth difficulties, resulting in autism.

Immunopathogenesis and autism

Stubbs (1976) found that a group of autistic children did not have rubella antibodies despite having a history of vaccination. This finding suggests that autistic children may lack the immune response to the rubella antigen, and implies a dysfunction in the immune system of autistic children. Stubbs et al. (1977) also measured T-cell stimulation by using phytohaemagglutinin (PHA) and B

lymphocyte responsiveness to pokeweed mitogen (PWM) of 12 children with autism. It was shown that the autistic group had a depressed lymphocyte transformation response to PHA as compared to the normal controls, suggesting that children with autism have a relative T-cell deficiency.

Weizman et al. (1982) examined a cell-mediated autoimmune response toward human MBP by the macrophage inhibition factor test in autistic subjects. Thirteen of 17 autistic subjects showed inhibition of macrophage migration, whereas none of the controls demonstrated such a response. The results indicated that there may exist a cell-mediated immune response to brain tissue in autistic patients.

In 1986, Warren et al. confirmed an earlier finding by Stubbs et al. (1977) that lymphocytes from autistic subjects have a defective response to the T-cell mitogen PHA and the B-cell mitogen PWM. A further study by this group demonstrated a lower percentage and number of CD4+ T cells, a lower number of CD4+CD45RA+ T cells, and a lower percentage and number of total lymphocytes in autistic subjects as compared to siblings and normal controls (Warren et al., 1990b).

Neuropathogenesis in autism

Some investigators have reported that brainstem dysfunctions that severely disrupt the development of many higher cognitive functions may be involved in autistic

children (Ornitz, 1983). Williams et al. (1980) examined the cerebral cortex of four individuals who showed prominent autistic features. They found subtle changes to the dendrites and spines of layer V pyramidal neurons of the mid-frontal gyrus in two patients. They also found a reduced density of Purkinje cells in a familial case.

The neuron-anatomical findings in autism from Yates (1984) show that many of the functional impairments constantly observed in autistic children suggest a damage to the left hemisphere of the brain. Bauman and Kemper (1985) performed an autopsy on the brain of a man with autism who died by drowning. They found a marked loss of Purkinje cells and some loss of granule cells in the neocerebellum.

Ritvo et al. (1986) studied the cerebellum of four patients and the pathology results showed that one patient had microgyria of the occipital and temporal lobes and total Purkinje cell counts of each of the four patients were significantly lower than those of comparison cases.

Autoimmune disorder and autism

In 1971, Money et al. reported that infantile autism and autoimmunity occurred coincidentally in a family and postulated that autoantibodies may affect the central nervous system. Sullivan (1975) found an increased incidence of the autoimmune disorder rheumatoid arthritis in families of autistic children, and Raiten and Massaro also found the same

situation in 1986. Stubbs (1987) noted the occurrence of an autoimmune disorder, systemic lupus erythematosus (SLE), in a mother of an autistic child. They also found several other mothers of autistic children having a presumed diagnosis of SLE. Todd and Ciaranello (1985) found that 9 of 30 autistic children had antiserotonin autoantibodies to brain serotonin receptors, in contrast to 0% in groups of control children.

Singh et al. (1991) demonstrated that the soluble cytokines interleukin-2 (products of activated immunocytes) and T8 are increased in the sera of children with autism. These cytokines have also been found in other autoimmune diseases such as systemic lupus erythematosus and multiple sclerosis (Huang et al., 1988; Trotter et al., 1988).

Warren et al. (1991) studied the frequency of the null allele at the complement C4B locus in autistic subjects and their siblings. They found that there is an increased phenotypic frequency of the C4B null allele in autistic subjects and mothers, whereas the fathers had normal frequencies of this null allele. In a subsequent study, they found that inheritance of the null allele is associated with reduced C4B serum levels, which may be allowing an infectious agent or immune complex to persist, resulting in prolonged immunological stimulus and triggering of an autoimmune reaction (Warren et al., 1994). Warren et al. (1992) demonstrated that autism, like other autoimmune diseases, also exhibits associations with particular alleles of the

major histocompatibility complex (MHC), located on chromosome 6. According to the above information, one can propose that some cases of autistic disorders may be associated with an autoimmune disorder.

Much evidence indicates that heat-shock proteins have a role in autoimmunity. Increased antibody levels to HSP cognates have been observed in different autoimmune disorders such as antibodies to HSP65 in rheumatoid arthritis and HSP70 in systemic lupus erythematosus (Bahr et al., 1988; Minota et al., 1988; Tsoulfa et al., 1989).

The nature of HSP

HSPs, a large family of proteins highly conserved between species, are synthesized by prokaryotic and eukaryotic cells. Some families of HSPs are induced by a variety of insults that promote protein denaturation and misfolding, while others play vital roles in normal cell function and are synthesized constitutively. Both of these types, induced and constitutively synthesized HSPs, are closely related in amino acid sequence.

The physiological functions of HSPs include the following: First, HSPs bind to other proteins and modify their destiny and function (Craig, 1985; Rothman, 1989). For example, HSP90 binds to steroid receptors, preventing the interaction between steroid receptors and nuclear DNA until the steroid hormone is bound. Without the presence of HSP90,

steroid receptors would interact with nuclear DNA independently, which would cause the premature and senseless interaction between steroid receptors and DNA (Sanchez and Housley, 1987). Second, HSPs have the ability to help the folding, unfolding, and translocation of polypeptides as well as the assembly and disassembly of oligomeric protein complexes (Cheng et al., 1989). The disassembly of oligomeric complexes and the unfolding of polypeptides occur under stress conditions such as high temperature and the reactive oxygen metabolites that frequently occur during the course of inflammation (Polla, 1988). It is probably a major role for HSP to prevent or reverse those events.

The major HSPs of most organisms can be classified by their sizes. They fall into size classes of approximately 80 to 90, 68 to 75, and 15 to 30 kilodaltons. Among these, the HSP70 proteins are a major group that account for 1% of total cellular protein (Pelham, 1986) and are extremely conserved within species. The HSP70 family consists of five different, but structurally and immunologically related proteins: (1) a constitutive 73kDa protein; (2) a stress-inducible 72kDa protein; (3) a stress-inducible 73kDa protein; (4) a constitutive 74-78kDa protein present in the endoplasmic reticulum; and (5) a 75-kDa member present in the mitochondria (Lindquist, 1986; Schlesinger, 1986). The inducible HSP70 proteins are thought to aid in protein repair (Pelham, 1986), while the constitutive HSP70 proteins are

known to participate in disassembly of clathrin vesicles (Chapel et al., 1986) and in assembly of immunoglobulin molecules.

HSP60 is the first mitochondrial component known to be essential in the process of folding and assembly of proteins imported into the mitochondria (Ostermann et al., 1989). It is a nuclear-coded, constitutively expressed heat-shock protein existing in the mitochondrial matrix. The HSP60 equivalent has been found in mitochondria from several sources. Since HSP60 can assist in oligomeric protein assembly, the loss of HSP60 function causes the failure of precursor proteins to assemble into functional proteins.

More recently, HSP70 and HSP60 from several species of parasites and bacteria have been shown to be antigenic (Bianco et al., 1986). As mentioned before, a host may experience stress induced by its predators, which may lead to increased HSP synthesis. Due to the increased amount of HSP, or perhaps for other reasons, HSPs become prominent antigens that trigger a major portion of the immune repertoire.

Antibodies against HSP cognates have been found in infections with many parasites (Ternynck et al., 1991). It is not clear whether this type of immunity can confer the protection against microbial pathogens. However, it has been proposed that this immunity adds to resistance at early stages of infections (Kaufmann, 1990).

HSP and autism

Since members of the HSP70 family are highly conserved at the amino acid level among eukaryotes and prokaryotes (Lindquist, 1986; Pelham, 1986), it has been suggested that the HSP70 family may play a role in autoimmunity. The antibodies induced against microbial HSP70 may crossreact with HSP70 from the host, leading to the development of autoimmune disorders. Furthermore, it has been demonstrated that three HSP70 genes are mapped in the human major histocompatibility complex (MHC) on chromosome 6 between the loci for complement components and the tumor necrosis factor (Sargent et al., 1989; Romano et al., 1989; Milner and Campbell, 1990). The MHC is a group of closely linked loci coding for surface molecules involved in many aspects of the immune response. The level of DNA polymorphism in human MHC is very high, creating many allelic patterns within MHC genes. It has been found that a combination of specific MHC alleles (extended haplotype) occurs more often than normal in subjects with certain autoimmune diseases (Alper et al., 1989). Warren et al. (1992) recently reported that one particular combination of MHC alleles B44-SC30-DR4 (HLA-B, BF, C2, C4A, C4B null allele, HLA-DR4) was increased by almost six-fold in autistic patients as compared with normal subjects. Therefore, they suggested that a gene somewhere within the extended haplotype is associated with manifestation of some cases of autism. Since the gene of the

HSP70 family has been mapped close to the extended haplotype, it is conceivable that HSP70 is responsible for the development of the neurobehavioral symptoms in autism.

Another important member in the HSP family is HSP60. Jones et al. (1993) identified that 19 known autoantigens are similar to regions of this HSP, and the regions of sequence similarity between them occurred at sites of high potential antigenicity. Also, the HSP60 is highly conserved within most organisms. Therefore, HSP60 has been associated with several autoimmune diseases. For example, a region (155-175) of human HSP60 is similar to the region (98-118) of a DQ alpha peptide. In primary biliary cirrhosis (PBC), the autoimmune destruction of HLA class II-bearing bile duct cells may be due to the similarities between T-cell epitopes in PBC-associated antigens and HLA-DRα (Burroughs et al., 1992).

Anti-MBP antibody and autism

Abnormal immune response to MBP has been found to be associated with multiple sclerosis (Waksman, 1981) and encephalitis (Desai et al., 1994). In 1982, Weizman et al. found that some autistic subjects make inappropriate cell-mediated immune response toward a component of brain myelin, MBP. In addition, Singh et al. (1993) examined the possibility of humoral-mediated autoimmune response toward

myelin basic protein in the syndrome of autism. Approximately 58% (19 of 33) of sera from autistic children were found to be positive for MBP antibodies. Moreover, Musiek et al. (1984) suggested that tardy or incomplete myelination in the corpus callosum is the basis of auditory processing problems in some learning disability children. McClelland et al. (1992) also reported a maturational defect in myelination within the brain-stem in 11 autistic children. Therefore, it is possible that an immunological invasion (i.e., autoantibodies) occurred after a viral infection, prenatally or postnatally, resulting in poor myelination or abnormal function of the neuron-axon myelin. Thus it is conceivable that anti-MBP antibodies may be associated with the development of neurobehavioral problems in a subgroup of the autistic subjects.

General principles of assay methods

Study of anti-HSP60 antibodies. The method used for detecting HSP60 antibody is the indirect solid-phase enzyme-linked immunosorbent assay (ELISA). In this assay, antigen is immobilized onto the surface of a microtiter well, and patient and control plasmas are added to react with the absorbed antigens. The unreacted molecules are washed away, and an enzyme-labeled anti-human immunoglobulin secondary antibody is added. Finally, color is developed with addition of a substrate.

The specificity of the HSP60 antibody assay results are confirmed using a competitive ELISA and an antibody competition assay. In the competitive ELISA, the positive plasmas (having a purportedly high antibody level to HSP60) are first incubated with the soluble HSP60 antigen. If the plasma contains the specific antibody, this antibody will be absorbed onto the soluble antigen (the soluble antigen used is the same as the antigen coated on the solid phase). After the adsorption process, part of the solution (plasma-antigen) is transferred to the corresponding wells of an antigen plate. The enzyme-linked anti-human Ig is then added to the wells and the plate is developed by the addition of substrate to each well. Since the antibody specific to the antigen will have been removed by the adsorption process, the absorbance values for the wells (sample wells) containing competing antigen would be expected to be less than those of wells (control wells) without competing antigen. The wells without competing antigen should have the maximal reading of optical density (OD).

In the antibody competition assay, positive plasmas are mixed with rabbit anti-HSP60 polyclonal antibody and added to the antigen (HSP60)-coated well. Control wells contain positive plasmas alone. If the antibodies compete for the binding to HSP60, the absorbance readings for the sample wells will be less than those of control wells.

Study of anti-HSP70 antibodies. The method for detecting HSP70 antibodies is the same as the method used above for detection of the HSP60 antibodies. The specificity of the reactivities is also studied with a competitive ELISA. This assay is similar to the competitive ELISA described above for HSP60 antibodies except that two kinds of soluble HSP70 antigens, obtained from different sources, are used. One of the soluble antigens is the same as that used to coat the plate. The other antigen is obtained from a different vendor.

Investigation of crossreactivity between patient anti-HSP60 antibodies and anti-MBP monoclonal antibodies. Sharing of epitopes between patient antibodies and anti-MBP monoclonal antibodies is examined by using an antibody competition assay. This assay is similar to the antibody competition assay described above except that it uses two kinds of anti-Ig secondary antibodies. In two microtiter plates, anti-HSP60 antibody in patient plasma is mixed with anti-MBP monoclonal antibody for competing binding to HSP60 coated in the plates. In the first plate, the addition of anti-human polyvalent immunoglobulins conjugated with alkaline phosphatase is used to measure the level of anti-HSP60 antibodies from patient plasma which have bound to the coating antigen. The wells without competing antibody are the control wells which will have the maximal reading of OD. If the anti-MBP monoclonal antibody can compete with anti-

HSP60 antibody for binding to HSP60 antigen, the OD values of the wells with competing antibody will be less than those of wells without competing antibody. In the second plate, the conjugate anti-mouse IgG is added to detect the amount of anti-MBP monoclonal antibodies which have bonded with the coating antigen. The control wells contain the anti-MBP monoclonal antibody plus coating antigen. If the anti-HSP60 antibody competes with anti-MBP monoclonal antibody for binding to the antigen, the OD values of the sample wells will be lower than those of control wells.

CHAPTER III

MATERIALS AND METHODS

Experimental subjects

This study included 41 autistic subjects (7 females and 34 males) ranging in age from 3 to 27 years from whom plasmas were obtained and stored in conjunction with previous studies carried out in the Biomedical Laboratory of the Center for Persons with Disabilities. All of the patients met DSM-IIIR criteria for autism as evaluated by at least one psychiatrist and confirmed by a psychologist. They had no medical problems or sign of infection and/or allergies at the time blood samples were collected. Thirty-two healthy, age-matched controls (9 females and 23 males) were also studied who were living in the same geographical area (northern Utah) as the autistic patients. All subjects were of northern European ancestry.

Plasma preparation

Blood samples were obtained by venipuncture during early morning hours via informed consent procedures. The plasma was separated from the blood by centrifugation at 2000 rpm for 15 minutes, aliquoted, and stored at -70° C until used.

Plasma standard for assays

In order to conduct this investigation, it was necessary to perform the ELISA on several different days. Therefore, to control for day-to-day variation, a plasma sample known to have antibodies against HSP60 or HSP70 (standard plasma) was included each time the assay was performed.

Sources of HSP60 and HSP70

The HSP70 (Sigma, St Louis, MO.) was purified from bovine brain following homogenization with Tris-HCL buffer (buffer A) containing 2-mercaptoethanol. After centrifugation the supernatant was fractionated with ammonium sulfate. Following centrifugation and dialysis, the sample was chromatographed on a DEAE-cellulose column. The proteins were eluted with a salt gradient and fractions at 0.1-0.2 M NaCl were pooled, and further purified by an ATP-agarose column.

The HSP70 (StressGen Victoria, B.C., Canada) was purified from fresh bovine brain. The method of purification follows the techniques described by Schlossman et al. (1984). The HSP60 (Epicentre Technologies, Madison, WI) was purified from E. coli bearing plasmid pOF39. Cultures of E. coli were grown to late log phase at $37^{\circ}C$, collected by centrifugation at $4^{\circ}C$, and resuspended in ice-cold Tris-saline containing protease inhibitors. After lysing by sonication, the suspension was centrifuged and fractionated by ammonium

sulfate precipitation. The ammonium sulfate cut was resuspended in Tris-saline and applied to a gel-filtration column. Fractions containing proteins were pooled, diluted with distilled water, and applied to a Q-Sepharose column equilibrated with bis-Tris-HCL and KCL. HSP60 was eluted with a KCL gradient and the protein was further purified by Mono-Q FPLC.

Buffers

1. Washing buffer: phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBS-T, pH 7.4) was used for all washing procedures of ELISA plates.

2. Dilution buffer: PBS containing 0.05% Tween 20 and 1% bovine serum albumin (BSA) (PBS-T-BSA) was used for diluting antibodies, human plasma, and enzyme-labeled reagents.

3. Blocking buffer: PBS containing 0.05% Tween 20 and 5% bovine serum albumin.

4. Coating buffer: Carbonate buffer, pH 9.6

5. Substrate buffer: 0.05M diethanolamine buffer, pH 9.8, containing 1 mM $MgCl_2$.

Study of anti-HSP60 antibodies

The optimal concentrations of antigen and antibody for all assays were determined by checkerboard titrations. Wells of 96-well microtiterplates (E.I.A.II Plus microtitration plates from Linbro, McLean, VA) were coated with 100 µl

(1 µg/ml) antigen, HSP60, in carbonate buffer (pH 9.6) and incubated overnight at 4°C (the coating antigen concentration used in all assays was the same). Excess antigen was decanted and the wells were washed with PBS-T. The unbound sites in the wells were coated with proteins of the blocking solution (100 µl) at 37°C for one hour. The wells were washed with PBS-T and duplicates of 100 µl of patient and control plasma diluted 1:50 and 1:100 in dilution buffer were added to test wells. Four controls were used in each assay including a coated solid-phase well with a negative plasma, a coated well with a standard plasma, an uncoated well with a standard plasma, and a coated well with all reactive reagents. Plates were incubated overnight at 4°C. After washing, 100 µl of goat-anti-human polyvalent antibody (Sigma, St. Louis, MO) conjugated with alkaline phosphatase in 1:10,000 dilution (the conjugated antibody concentration used in all assay were the same) was added to each well and the plate was incubated at 4°C overnight. Following five washes, the enzyme-substrate reaction was developed by the addition of 100 µl p-nitrophenol phosphate (1 mg/ml in substrate buffer) (Sigma, St. Louis, MO) to each well. Absorbance was measured at 405 nm with a BIO-RAD microplate reader.

In the competitive ELISA, 100 µl (1 µg/ml) of HSP60 antigen was pipetted into each well of the microplate and incubated at 4°C overnight. An equal volume of positive

plasma (1:25 dilution) and soluble antigen (4 µg/ml HSP60 in dilution buffer) were incubated for 90 minutes at 37°C in a siliconized tube in a humid environment. Siliconization with dichlorodimethylsilane (Sigma) was used to prevent protein from sticking to the surface of tube. A control tube containing the positive plasma (1:50 dilution) was incubated in the same environment as the sample tube. Blocking solution was added to the plate and incubated for one hour at 37°C. The plasma-antigen solution (100 µl) and plasma only (100 µl) were incubated with coating antigen overnight at 4°C. The development of the ELISA with the conjugated antibody, addition of the substrate, and absorbance measurement were the same as the procedures used for detection of the HSP60 antibodies.

The procedures for antibody competition assay were similar to the procedures used in the competitive ELISA except that anti-HSP60 polyclonal antibodies and patient plasma were incubated together. Duplicate 50 µl volumes of a 1:100 dilution of rabbit anti-HSP60 polyclonal antibodies (Epicentre, Madison, WI) were added to the microtiter plate and allowed to bind for one hour at room temperature. Without washing, 50 µl of a 1:25 dilution of positive plasma was added in duplicate to the plate containing antigen and anti-HSP60 polyclonal antibodies and incubated at 4°C overnight. The control wells contained HSP60 as an antigen and 100 µl of a 1:50 dilution of positive plasma.

Study of anti–HSP70 antibodies

The method for detecting HSP70 antibodies in patient and control plasmas was the same as that used in measuring the HSP60 antibodies. The specificity of the positive plasmas was studied by a competitive ELISA which was similar to that mentioned above for HSP60 antibodies. Microplates were coated with HSP70. The next step was separated into two parts. In the first part, an equal volume of positive plasma (1:25 dilution) and soluble HSP70 coating antigen (Sigma, St. Louis, MO) (4 µg/ml in dilution buffer) were incubated in a siliconized tube for 90 minutes at 37°C in a humid environment. In the second part, soluble HSP70 antigen at concentration of 4 µg/ml (StressGen, Victoria, B.C., Canada) was incubated with a positive plasma (1:25 dilution) in a siliconized tube. A tube containing the positive plasma (1:50 dilution) which gave the maximal OD was incubated in the same environment as the sample tubes.

Investigation of crossreactivity
between anti–HSP60 antibodies and
anti–MBP monoclonal antibodies

The procedures for this study were similar to the antibody competition assay described above except that two kinds of secondary conjugated antibodies were used. In one assay, duplicate 50-µl volumes of 1:100 dilution of

monoclonal antibody with specificity for MBP (Boehringer Mannheim Biochemical, Indianapolis, IN) were added to the microtiter plate and allowed to bind for one hour at room temperature. Without washing, 50 µl of 1:25 dilution (in dilution buffer) of positive plasma was added in duplicate to the plate containing HSP60 antigen and anti-MBP monoclonal antibody and incubated at 4°C overnight. The plate was washed five times in PBS-T, and 100 µl of 1:10,000 dilution of alkaline phosphatase conjugate of anti-human polyvalent antibodies was added into plate. The control wells contained coating antigen (HSP60) and positive plasma (1:50 dilution). The second procedure was similar to that of the first assay except that the conjugated antibody used in the second assay was anti-mouse IgG conjugated with alkaline phosphatase (1:2000 dilution in dilution buffer). The control wells contained coating antigen, HSP60, and anti-MBP monoclonal antibodies (1:200 dilution).

Statistical analysis

The OD from the assays was expressed as a ratio of the standard in each plate to adjust for the deviated results within each plate. Direct absorbance values were also used as an additional method to evaluate data. Statistical comparisons between autistic and control groups were made using Student's t test. Comparisons were also performed between autistic males and control males, and between autistic females and controls. Due to the limited number of

autistic females and control females, the comparison was made between autistic females and total controls rather than control females.

The correlation coefficient was estimated by simple regression analysis.

In antibody competition assays, the percentage of inhibition was calculated by the following formula:

$$\% \text{ inhibition} = \frac{\text{OD of wells without competing Ab} - \text{OD of wells with competing Ab}}{\text{OD of wells without competing Ab}} \times 100$$

CHAPTER IV

RESULTS

Study of anti-HSP70 antibodies

Circulating anti-HSP70 antibody levels as estimated by the OD values in plasma samples calculated as a percentage of OD values in standard plasma are shown in Figures 1 and 2. Values in autistic subject plasmas diluted 1:50 were significantly elevated (median 29.24; range 8.79-100; $P=0.0148$) as compared with those of the normal controls (17.37; 4.37-70.048) (Fig. 1). Similar results were obtained with plasma diluted 1:100 (Fig. 2). In a comparison of the plasmas of the autistic males and control males, OD ratios were significantly increased ($P=0.0285$) in the former (Fig. 3). The OD ratios of plasmas of the autistic females were not significantly elevated when compared with normal female controls, but were significant ($P=0.0293$) if compared with all (male and female controls) (Fig. 4).

A direct comparison (without standard adjustment) of patient plasmas diluted 1:50 (Fig. 5) and 1:100 (Fig. 6) gave results similar to those obtained using the OD ratio described above. Significant increases in OD values in patient plasmas were seen in both analyses.
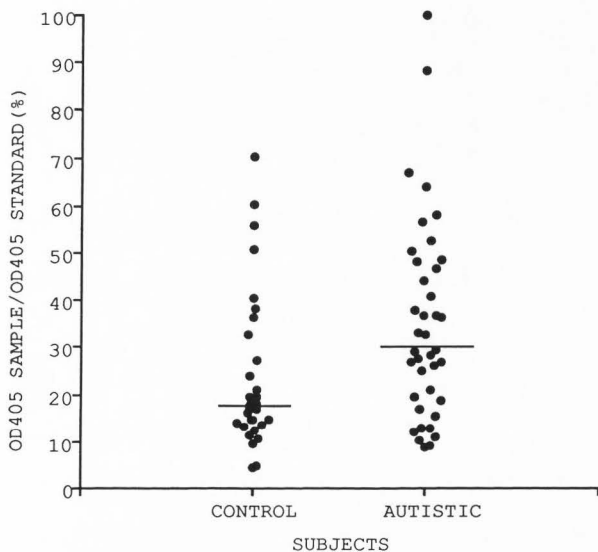
Fig. 1. Antibody reactivity to HSP70 in plasmas of autistic subjects and normal controls. Shown are OD ratios (OD405 sample/OD405 standard) and median of plasmas diluted 1:50. Plasmas of autistic subjects had significantly (*P*=0.0148) elevated ratios.
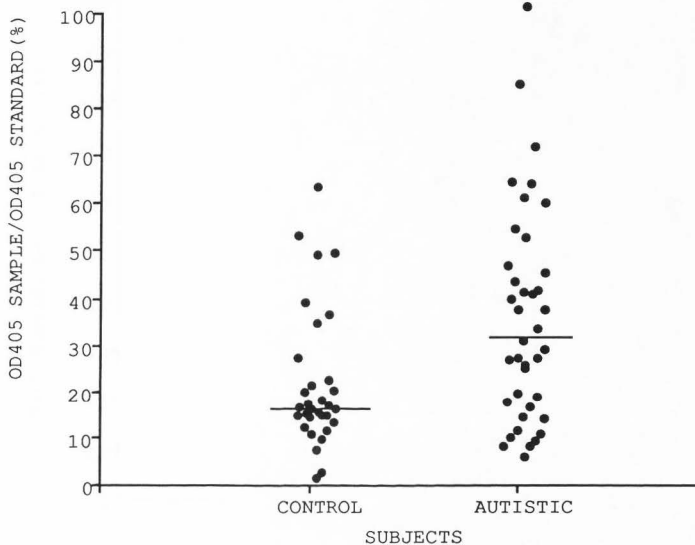
Fig. 2. Antibody reactivity to HSP70 in plasmas of autistic subjects and normal controls. Shown are OD ratios (OD405 sample/OD 405 standard) and median of plasmas diluted 1:100. Plasmas of autistic subjects had significantly (*P*=0.0048) elevated ratios.

Fig. 3. Antibody reactivity to HSP70 in plasmas of autistic male subjects and normal male controls. Shown are OD ratios (OD405 sample/OD 405 standard) and median of plasmas diluted 1:50. Plasmas of autistic male subjects had significantly (*P*=0.0285) elevated ratios.
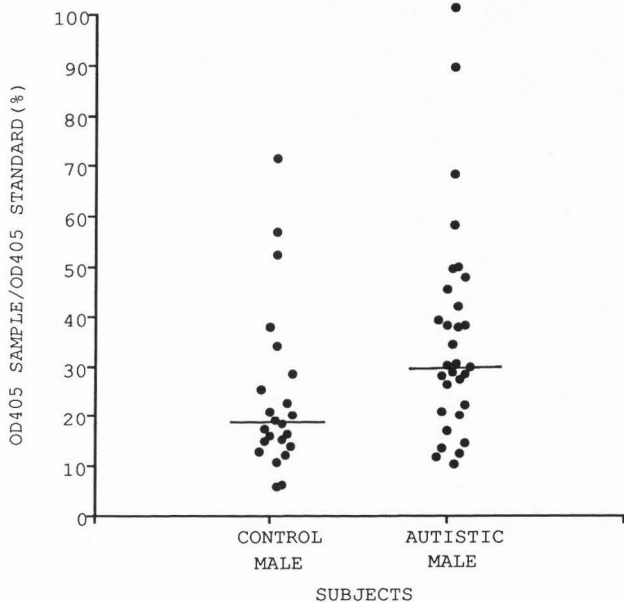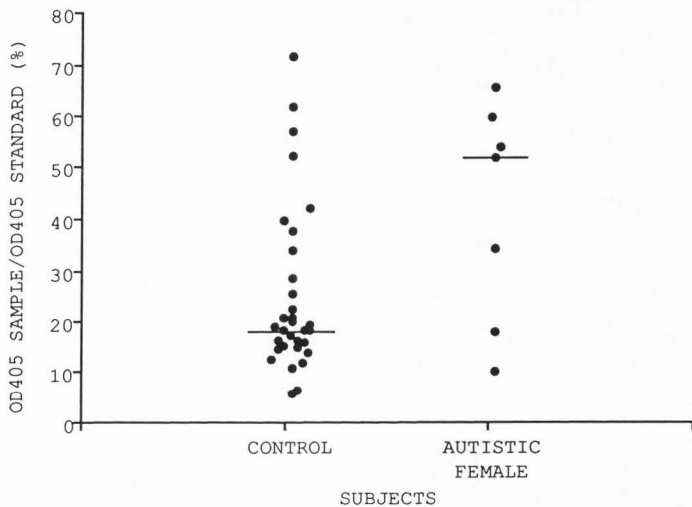
Fig. 4. Antibody reactivity to HSP70 in plasmas of autistic female subjects and normal controls. Shown are OD ratios (OD405 sample/OD405 standard) and median of plasmas diluted 1:50. Plasmas of autistic female subjects had significantly (P=0.0293) elevated ratios.

Fig. 5. Antibody reactivity to HSP70 in plasmas of autistic subjects and normal controls. Shown are absorbance values (median) from plasmas diluted 1:50. Plasmas of autistic subjects had significantly (*P*=0.0111) elevated values.
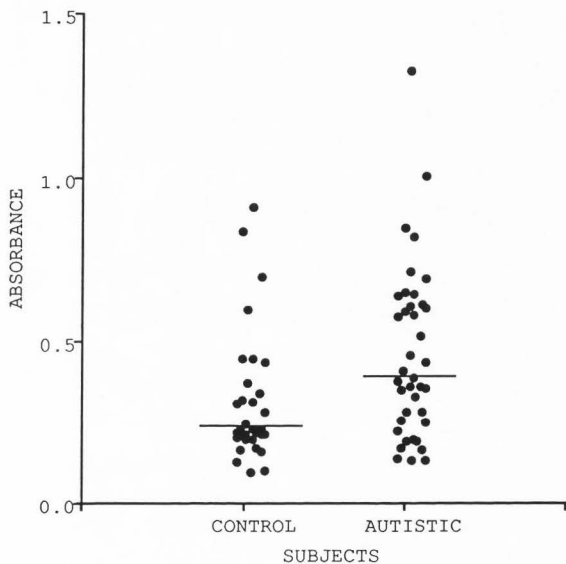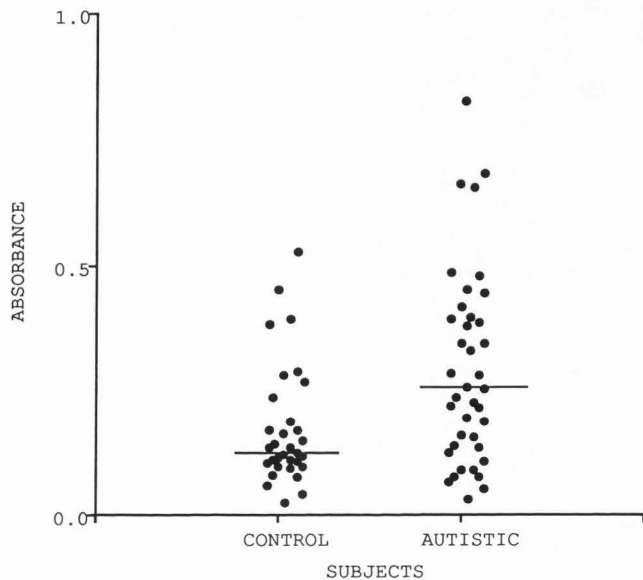
Fig. 6. Antibody reactivity to HSP70 in plasmas of autistic subjects and normal controls. Shown are absorbance values (median) from plasmas diluted 1:100. Plasmas of autistic subjects had significantly (*P*=0.0041) elevated values.

The specificity of the results was studied in two different competitive assays. In the first, the plasmas giving high OD values were incubated with HSP70 (purchased from Sigma) in solution before pipeting them into antigen coated wells. The absorbance values obtained with these plasmas were significantly lower ($P<0.0001$) than those found with plasmas not incubated with the antigen solution (Fig. 7). The second competitive assay was the same as the first one except that the soluble antigen was purchased from StressGen. The results were similar ($P<0.0003$) to those found with antigen obtained from Sigma (Fig. 8).

Study of anti-HSP60 antibodies

The levels of anti-HSP60 antibodies in the plasmas of the 41 autistic subjects (median 19.24; range 0-100; $P=0.1303$) were not significantly increased as compared with those (10.63; 0.88-71.33) of normal healthy controls (Fig. 9 and 10). Nevertheless, 13 out of 41 autistic subjects' (32%) plasmas had significantly increased antibody levels above the 95% confidence limits of the control group. The antibody levels in the plasmas of autistic males were significantly higher ($P=0.0339$) than those of normal controls (Fig. 11). In contrast, the mean OD ratios between the autistic (28.1) and control females (29.1) were similar (Fig. 12).

Fig. 7. Antibody reactivity to HSP70. Absorbance values from the wells without competing antigens (white bars) and with competing antigens (black bars) in plasmas of autistic subjects (#1, 2, 3, 4, & 5) and in plasmas of controls (#6, & 7). The preparation of HSP70 antigen used to adsorb antibodies was different from that of HSP70 used to coat the plate. The absorbance values from the wells with competing antigens were significantly lower (*P*<0.0003) than those of wells without competing antigens.
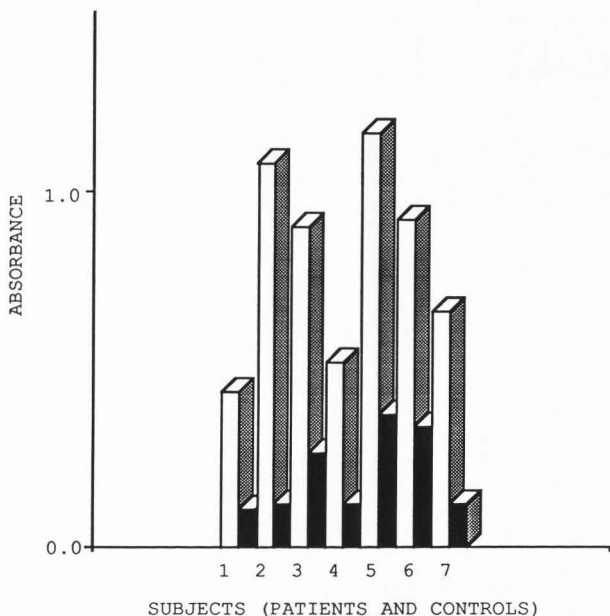
SUBJECTS (PATIENTS AND CONTROLS)

Fig. 8. Antibody reactivity to HSP70. Absorbance values from the wells without competing antigens (white bars) and the wells with competing antigens (black bars) in plasmas of autistic subjects (#1, 2, 3, 4, & 5) and in plasmas of controls (#6, & 7). The preparation of HSP70 antigen used to adsorb antibodies was the same as that of HSP70 antigen used to coat the plate. The absorbance values from the wells with competing antigens were significantly lower ($P<0.0001$) than those of wells without competing antigens.

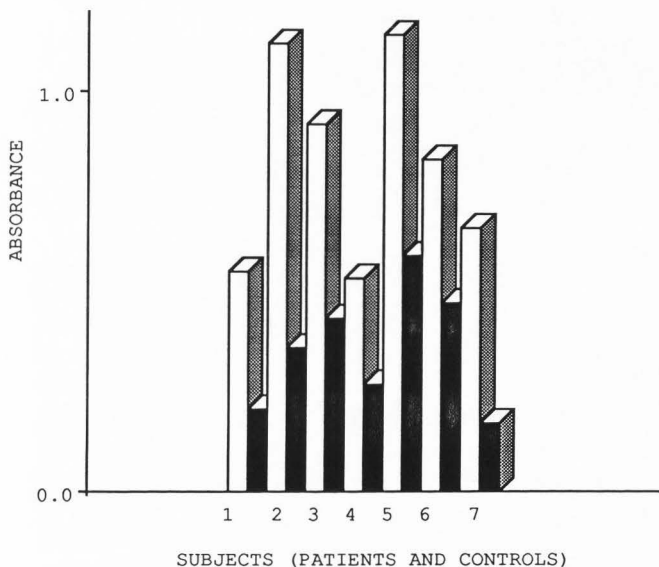**Fig. 9.** Antibody reactivity to HSP60 in plasmas of autistic subjects and normal controls. Shown are OD ratios (OD405 sample/OD405 standard) and median of plasmas diluted 1:50. No significantly (*P*=0.1303) different findings were seen.

Fig. 10. Antibody reactivity to HSP 60 in plasmas of autistic subjects and normal controls. Shown are OD ratios (OD405 sample/OD405 standard) and median of plasmas diluted 1:100. No significantly (*P*=0.154) different findings were seen.

Fig. 11. Antibody reactivity to HSP60 in plasmas of autistic male subjects and normal male controls. Shown are OD ratios (OD405 sample/OD405 standard) and median of plasmas diluted 1:50. Plasmas of autistic male subjects had significantly (*P*=0.0339) elevated ratios.
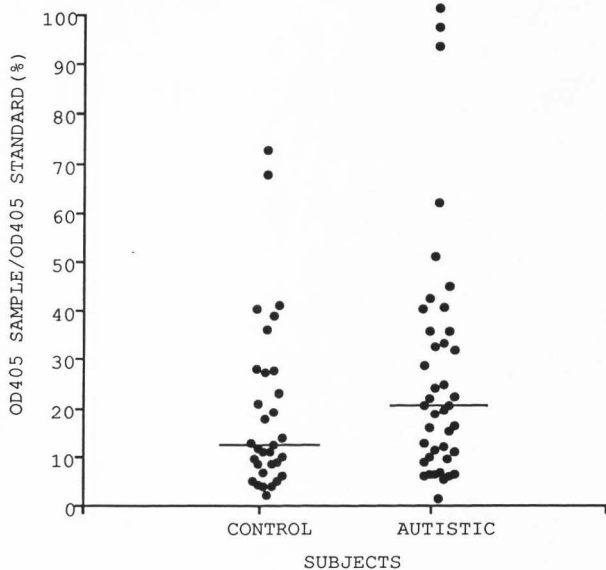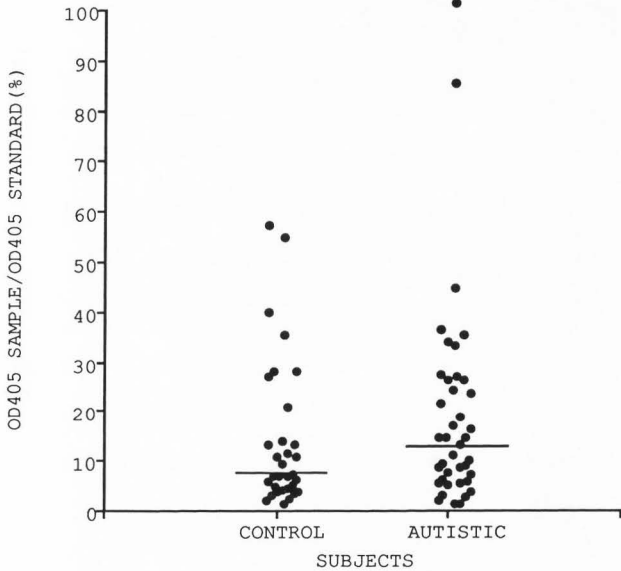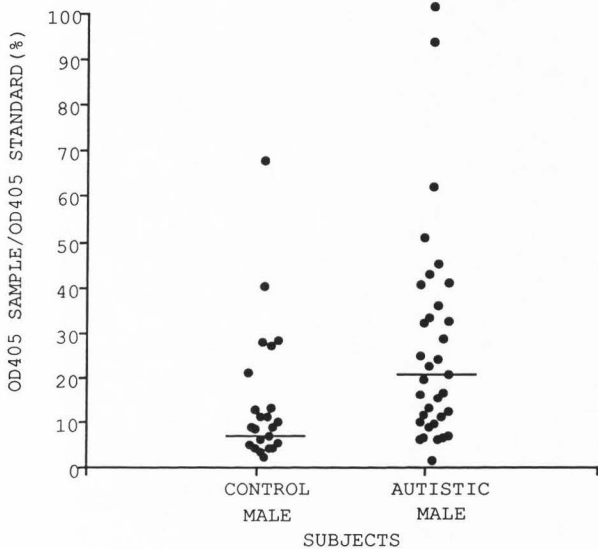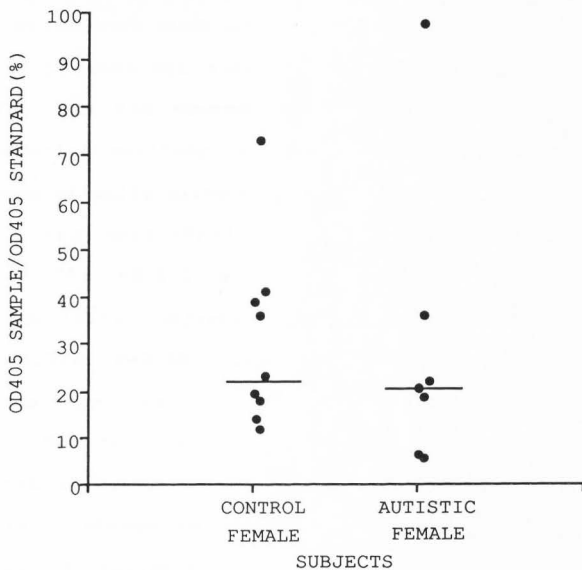
Fig. 12. Antibody reactivity to HSP60 in plasmas of autistic female subjects and normal female controls. Shown are OD ratios (OD405 sample/OD405 standard) and median of plasmas diluted 1:50. Plasmas of autistic female subjects did not have significantly (*P*=0.933) elevated ratios.

The specificity of these results was also studied by competitive assays. In the first assay, the OD values obtained with plasmas incubated with the antigen (HSP60) solution were much lower ($P<0.0001$) than the OD values found with plasmas not incubated with the antigen solution (Fig. 13). In the second assay, OD values of the wells with competing antibody were significantly ($P<0.0335$) lower than those of wells without competing antibody (Table 1).

The anti-HSP70 antibody levels were not correlated ($r=0.105$; $P=0.523$) with anti-HSP60 antibody levels in plasmas of autistic subjects. Likewise, there was no correlation ($r=0.024$; $P=0.897$) between these antibodies in plasmas of normal controls.

The correlation between ages of autistic subjects and antibody levels to HSP60 and HSP70 was also compared. The results showed no correlation ($r=0.234$; $P=0.0.118$) between ages of autistic subjects and the levels of anti-HSP60 antibodies. Also, the ages of autistic subjects were not correlated ($r=0.104$; $P=0.547$) with the levels of anti-HSP70 antibodies.
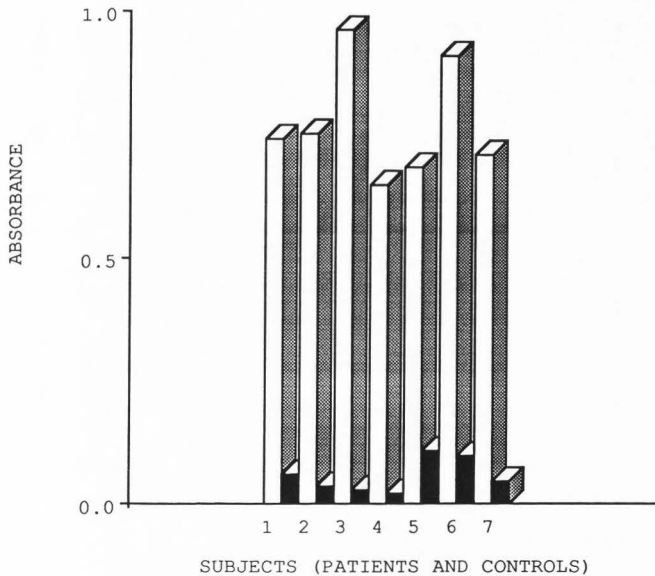
Fig. 13. Antibody reactivity to HSP 60. Absorbance values from the wells without competing antigens (white bars) and the wells with competing antigens (black bars) in plasmas of autistic subjects (#1, 2, & 3) and in plasmas of controls (#4, 5, 6. & 7). The preparation of HSP 60 antigen used to adsorb antibodies was the same as that of HSP 60 antigen used to coat the plate. The absorbance values from the wells with competing antigens were significantly lower (*P*<0.0001) than those of wells without competing antigens.

Table 1. Specificity of patient and control plasmas[a] in
reactivity with HSP60

Subjects	Noncompetitive[b] OD	Competitive[c] OD	% inhibiton
1	0.431	0.137	68.21
2	0.332	0.200	39.76
3	0.258	0.128	50.39
4	0.209	0.109	57.92

a. Plasmas of autistic subjects (#1, 2, & 3) and normal
control (#4) diluted 1:50.

b. Patient or control plasma alone.

c. Patient or control plasma plus competing antibody.

d. The OD values were significantly lower (*P*<0.0335) in the
wells with competing antibody.

Investigation of crosseractivity
between anti–HSP60 antibodies
and anti–MBP monoclonal antibodies

In a first study of shared epitopes, patient plasmas yielding high OD values were allowed to compete with anti–MBP monoclonal antibodies for binding to HSP60 antigen coated in the plate. The control wells contained patient plasmas alone. The OD values of the wells with competing antibody were not significantly lower than those of wells without competing antibody (Table 2). In the second study, the sample wells were prepared the same way as those described in the first study except that anti–mouse Ig was added as an secondary antibody. The control wells contained anti–MBP monoclonal antibodies alone. Since the mouse anti–MBP monoclonal antibody did not bind to the coated antigen (HSP60) from E. coli, OD values for the wells with competing antibody and the wells without competing antibody were very low. No competition was found in the second study.

Table 2. Competition between patient plasma[a] and monoclonal
antibodies reactivity with MBP

Subjects	Noncompetitive[b] OD	Competitive[c] OD	% Inhitition
1	0.51	0.53	0
2	0.34	0.31	8.8
3	0.98	0.94	4.1

a. OD values from plasmas diluted 1:50.

b. Patient plasma alone.

c. Patient plasma plus monoclonal antibodies that were
reactive with myelin basic protein.

d. No significantly different findings were seen ($P<0.3486$).

CHAPTER V

DISCUSSION

Money et al. (1971) suggested that autism might be the result of the autoimmune mechanisms that damage the central nervous system. Several recent immunological studies have shown that autism shares certain features with established autoimmune diseases. For example, autism exhibits a greater concordance rate in monozygotic twins than in the same-sex dizygotic twins, a genetic predisposition factor that is also found in autoimmune diseases such as insulin-dependent diabetes and rheumatoid arthritis (Folstein and Rutter, 1988). Autistic disorder occurs more often in males. This gender factor is also common in ankylosing spondylitis, which occurs more often in males, and systemic lupus erythematosus (SLE), which occurs more often in females. Singh et al. (1991) found that the cytokines interleukin-2 and T8 are increased in the sera of autistic children; likewise, similar findings are also found in other autoimmune diseases such as multiple sclerosis (Trotter et al., 1988) and SLE (Huang et al., 1988). Many of the autoimmune diseases are associated with particular MHC haplotypes. For instance, ankylosing spondylitis is strongly associated with HLA-B27 antigen; juvenile rheumatoid arthritis is associated with HLA-DR5

antigen; and rheumatoid arthritis is associated with HLA-DR4 antigen (Stastny et al., 1983). Warren et al. (1992) found an association of an extended MHC haplotype B44-SC30-DR4 with autism and have suggested that a gene somewhere within the extended haplotype may be responsible for some cases of autism.

This study found a significant increase of anti-HSP70 antibody levels in autistic subjects. Four possible pathogenic mechanisms can be generated from these results. First, since three members of the HSP70 gene family have been mapped to the MHC class III region in man (Sargent et al., 1989; Milner et al., 1992) and members of HSP70 are known to be involved in immune response, one can imply that the HSP70 gene may contribute to immune genetic susceptibility to autism. Warren et al. (1991) also reported that the frequency of the C4B null allele is increased in autism and in a subsequent study found that the C4B null allele results in decreased plasma levels of the C4B protein in some autistic subjects (Warren et al., 1994). The products of the C4B gene are crucial in protecting against invading microorganisms. The C4B gene is also important because of its location in the middle of the extended MHC complex and its closeness to the HSP70 genes. It is not unreasonable that an allele of HSP70 gene may be inherited with the C4B null gene and may contribute to the susceptibility or development of autism.

Second, Pociot et al. (1993) studied the functional implication of the polymorphism in the heat-shock inducible 72kDa HSP gene in IDDM and showed that people who carry the Pst I 8.5-kb allele consistently had slightly lower expression than individuals who carry the 9.0-kb allele. Therefore, it is feasible that a subgroup of autistic subjects carries an allele that results in higher production of HSP70. In addition, autism has been associated with organisms causing gestational pathology, including prenatal rubella, cytomegalovirus, syphilis, and variolla (Desmond et al., 1970; Chess et al., 1971; Feldman et al., 1973; Knobloch and Pasmanick, 1974). In a normal cell (e.g., brain cell or nerve cell), heat shock proteins exist inside the cell compartments, such as cytoplasm and mitochondria. However, these proteins may be expressed on the cell surface after an insult by some stressors such as heat, viral infection (e.g., cytomegalovirus and rubella virus), etc. The increased expression of cell surface HSP70 may be considered foreign by the immune system, giving rise to a primary autoimmune reaction against HSP70, and may lead to the neurobehavior syndromes of autism.

Third, due to the extreme conservation of the primary structure of HSP70 and the expression of this protein in a number of organisms, it is possible that in autism anti-HSP70 antibody raised against a bacterial HSP70 epitope crossreacts

with brain cells or nerve cells containing similar epitope motifs with HSP 70.

Fourth, HSP70 might have something to do with autism, and the high antibody levels toward HSP70 might relate to autism or be a marker for autism. It is possible that HSP70 is required to keep repairing nerve or brain cells. Perhaps the high levels of anti-HSP70 antibodies modify or block the action of HSP70. Therefore, HSP70 may be unable to function properly. Such inactivation by anti-HSP70 antibodies could lead to some symptoms of autism.

On the other hand, some normal subjects had high levels of HSP70 antibodies in their plasma. The occurrence of these antibodies might be due either to some previous self-limited infections with certain organisms having immunogens homologous to HSP70 or to naturally occurring antibodies.

It is possible that immune responses to heat-shock proteins are normal, but other factors (e.g., MHC haplotype, environment, etc.) alter the normal immune responses to heat-shock protein. Several investigators have reported abnormal immune systems in autistic subjects. For example, the subjects have hyporesponses to the rubella antigen (Stubbs, 1976). In that light, it is possible that since both autistic subjects and normal controls do mount immune responses toward HSP70, the high antibody levels in plasmas of the autistic subjects might be the result of an abnormal immune system. It may also due to the defect in the

regulation of antibody response. Autistic subjects may make more antibodies toward HSP70 or their antibodies against HSP70 have a longer biological half life.

The specificity of the reactive plasma was studied using a competitive ELISA. In this assay, soluble HSP70 was used to remove antibodies specifically against HSP70 in plasmas. Since the purification technique for different preparations of HSP70 likely results in distinct impurities which might affect the results of this assay, two different preparations of HSP70 purchased from different companies were used in this study. The results suggested that the antibodies measured in this assay were directed against HSP70 and not the impurities.

Four controls were used in the study of anti-HSP70 antibodies. Among them, the wells containing standard plasmas alone were used to eliminate the false positive results due to the reaction between bovine serum albumin (BSA) in blocking solution and anti-BSA antibodies in human plasmas. Two autistic subjects were found to have antibodies against BSA; therefore, these samples were excluded from this assay. Nevertheless, it is possible that there are some antibodies against bovine proteins that can never be ruled out.

The levels of HSP60 antibodies in the plasmas of autistic subjects showed considerable individual variation and were not significantly increased above the mean antibody

levels of the normal control group. However, antibody levels in plasmas of 13 out of 41 autistic subjects were significantly elevated above the upper 95% confidence limits of the control subjects. In this subgroup of autistic subjects, further analysis of the specificity of antibodies will be required to verify reactivity against E. coli HSP60 and not contaminated E. coli proteins.

Since MBP shares sequence homology with human HSP60, and since HSP60 proteins are highly conserved within organisms, it is possible that antibodies against foreign HSP60 of microorganisms crossreact with human HSP60 and MBP on brain cells, leading to neurobehavior syndromes of autism. However, in this study, using a competition assay, no crossreactivity was found between the anti-MBP monoclonal antibodies and anti-HSP60 antibodies from the autistic patients. Conceivably the anti-MBP monoclonal antibodies used in this study were specific for an epitope that is not shared with HSP60 and would not be informative in assessing crossreaction with anti-HSP60 antibodies. It would be valuable in future research to study this competition assay by using polyclonal anti-MBP antibodies.

The finding in the study on anti-HSP antibodies in autistic patients, once again, suggests an association between autism and autoimmune disease. These results provide a stimulus for further research on HSP genes in autistic patients.

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