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## Circulating Antibodies to Thymic Antigens in Autism and Alzheimer's Disease

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CIRCULATING ANTIBODIES TO THYMIC ANTIGENS  
IN AUTISM AND ALZHEIMER'S DISEASE

by

Chih-Li Chen

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

of

MASTER OF SCIENCE

in

Biology  
(Microbiology/Immunology)

Approved:

UTAH STATE UNIVERSITY  
Logan, Utah

1992

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## CONTENTS

	Page
ACKNOWLEDGEMENTS . . . . .	ii
LIST OF FIGURES . . . . .	v
ABSTRACT . . . . .	vii
INTRODUCTION . . . . .	1
LITERATURE REVIEW . . . . .	3
Autism . . . . .	3
Genetic Implications and Autism . . . . .	4
Viral Infection and Autism . . . . .	5
Immunology and Autism . . . . .	6
Alzheimer's Disease . . . . .	8
Biochemical Abnormalities in AD Brains . . . . .	9
Immunology and AD . . . . .	11
Human Thymocytes . . . . .	14
Murine Thymocytes . . . . .	15
Thymic Epithelial Cells and Thymocytes . . . . .	16
The Complement System . . . . .	17
MATERIALS AND METHODS . . . . .	18
Statistical Methods . . . . .	18
Experimental Subjects . . . . .	19
Collection of Human Plasma Samples . . . . .	19
Collection and Preparation of Murine Thymocytes . . . . .	20
Detection of Antithymic Antibodies . . . . .	21
Proliferation of Thymic Cells . . . . .	22
Assay for Complement-Dependent Cytotoxic Activity . . . . .	23
RESULTS . . . . .	25
Detection of Antithymic Antibodies . . . . .	25
Proliferation of Thymic Cells . . . . .	32
Assay for Complement-Dependent Cytotoxic Activity . . . . .	32
Autistic Patient Plasma . . . . .	32
Alzheimer's Patient Plasma . . . . .	36
DISCUSSION . . . . .	54

CONCLUSIONS . . . . .	58
REFERENCES . . . . .	60

## LIST OF FIGURES

Figure	Page
1 Antithymic antibodies in autistic patients . . . . .	26
2 Antithymic antibodies in AD patients . . . . .	27
3 ELISA data for plasmas diluted 1:2.5 . . . . .	28
4 ELISA data for plasmas diluted 1:5 . . . . .	29
5 ELISA data for plasmas diluted 1:10 . . . . .	30
6 ELISA data for plasmas diluted 1:20 . . . . .	31
7 Proliferation using autistic patient plasma . . . . .	33
8 Proliferation using AD patient plasmas . . . . .	34
9 Untreated autistic patient plasmas . . . . .	35
10 Heat-treated autistic patient plasmas . . . . .	37
11 Complement-treated autistic patient plasmas . . . . .	38
12 Untreated AD patient plasmas . . . . .	39
13 Heat-treated AD patient plasmas . . . . .	40
14 Complement-treated AD patient plasmas . . . . .	41
15 Untreated plasmas diluted 1:64 . . . . .	42
16 Untreated plasmas diluted 1:128 . . . . .	43
17 Untreated plasmas diluted 1:256 . . . . .	44
18 Untreated plasmas diluted 1:512 . . . . .	45
19 Heat-treated plasmas diluted 1:64 . . . . .	46
20 Heat-treated plasmas diluted 1:128 . . . . .	47
21 Heat-treated plasmas diluted 1:256 . . . . .	48
22 Heat-treated plasmas diluted 1:512 . . . . .	49
23 Complement-treated plasmas diluted 1:64 . . . . .	50
24 Complement-treated plasmas diluted 1:128 . . . . .	51
25 Complement-treated plasmas diluted 1:256 . . . . .	52

vi

26      Complement-treated plasmas diluted 1:512 . . . . 53

ABSTRACT

Circulating Antibodies to Thymic Antigens  
in Autism and Alzheimer's Disease

by

Chih-Li Chen, Master of Science  
Utah State University, 1992

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

Abnormal T lymphocyte reactions in both autism and Alzheimer's disease (AD) have been reported. This research investigated the possibility that these abnormalities may involve circulating antithymic antibodies. Plasma samples from autistic patients, AD patients, and normal-matched controls were tested for reactivity against murine thymocytes.

In the first of 3 studies results of the enzyme-linked immunosorbent assay (ELISA) were statistically significant for binding ( $P < 0.001$ ) between antithymic antibodies in plasmas of AD patients and murine thymocytes. Binding ( $P < 0.05$ ) in low dilutions (1/2.5 and 1/5) of autistic patient plasmas was also observed. In the second study, plasmas of neither autistic nor AD patients significantly inhibited DNA synthesis of thymic cells in the presence of interleukin-1 (IL-1) and phytohemagglutinin (PHA). In the third study, no significant increases ( $P > 0.05$ ) in cytotoxic activities were detected

using AD patient plasmas and both untreated and heat-treated autistic patient plasmas. After further testing, these heat-treated plasmas diluted 1/64 and 1/128 had increased cytotoxicities ( $P < 0.05$ ) when rabbit complement was added, an indication that cytotoxicity of antithymic antibodies is complement dependent.

Therefore, circulating antithymic antibodies may be involved in abnormal T lymphocyte reactions in autism and AD. Since they probably do not act alone, future research should study these complex abnormalities using human thymocytes.

(73 pages)

## INTRODUCTION

Autism and Alzheimer's disease (AD) are characterized by disturbances in social, communicative, and cognitive abilities. The actual causes of autism and AD are still unknown. Evidence has accumulated over the past several years that links abnormalities of the immune system with both autism and AD. These immune abnormalities include decreased numbers of T lymphocytes, altered ratios of helper to suppressor T cells, and autoimmune mechanisms, involving both humoral and cell-mediated immunity.

In initial studies of the humoral immunity of autistic patients, some autistic children failed to produce specific antibodies following rubella vaccination (Stubbs, 1976). In contrast, high amounts of serum IgG3 subclass in AD patients were later reported (Singh & Fudenberg, 1989). IgG3 is the immunoglobulin which can most effectively activate the classical pathway of the complement system to damage tissues, such as the thymus. This tissue damage may then affect cell-mediated immunity, which depends on the thymus.

Studies of cell-mediated immunity in autistic patients revealed abnormal *in vitro* lymphocyte responsiveness to T cell mitogens (Stubbs, Crawford, Burger, & Vandenbark, 1977; Warren, Foster, Margaretten, & Pace, 1986; Singh & Fudenberg, 1988b). Moreover, a reduced number of total T (CD2+ cells) and B (CD20+ cells) lymphocytes and low percentages of CD4+ and CD45R helper T cells were found in some autistic children (Yonk *et al.*, 1990; Warren *et al.*, 1990).

In theory, at least, some of these abnormalities of cell-mediated immunity (T cell-derived) functions may be due to an autoimmune response, abnormal T cell differentiation and maturation, or a disturbed function of immunoregulatory T cells. The thymus gland, which is essential for the differentiation and maturation of T cells, and for the development of autoimmunity (Theofilopoulos, 1987), could conceivably be a target for the pathological and clinical manifestations seen in central nervous system (CNS) syndromes. In this regard, the finding of antithymic antibodies in sera of patients with schizophrenia (Heath, Krupp, & Byers, 1967; Coffey, 1983) represents an example of this type of autoimmune abnormality in a mental disorder. In addition, circulating antibodies to brain tissue antigens were found in patients with AD (Singh & Fudenberg, 1986) and autism (Singh, Fudenberg, Emerson, & Coleman, 1988). These brain antibodies, at least in patients with AD, cross-react with thymic cells (Singh & Fudenberg, 1988b), suggesting that the immunoreactive antigen might be a common antigen in the brain and thymus.

Based on the above considerations, the possibility of circulating autoantibodies against thymic tissue antigens (both thymocytes and epithelial cells) in autistic and AD patients was studied. Thymocytes from murine (strain C57BL/6, 6 to 10 weeks old) thymuses were collected. Plasma from autistic patients, AD patients, and normal-matched controls were prepared. The ELISA method was used to detect the

possible existence of thymocyte antigens that can bind plasma antibodies. The proliferative response of thymocytes was used to see if plasma antibodies can inhibit DNA synthesis of thymocytes in response to PHA. The complement-dependent cytotoxicity assay method was used to calculate the percentage of killing of thymocytes by plasma antibodies.

#### LITERATURE REVIEW

##### *Autism*

The syndrome of autism was first characterized by Kanner in 1943 in an attempt to describe disturbances of affective contact in early childhood. Children with this syndrome show disturbances in social relatedness, communicative skills, and certain information processing and cognitive abilities (Cohen, Paul, & Volkmar, 1986). Autism is manifested at birth, or shortly thereafter, and remains throughout the lifetime of the patient. Parents of autistic children are not always able to realize the aberrant nature of their infants. Sometimes they ignore it. Abnormalities may be evident from the first year and are always noticeable by 30 months of age (Folstein, & Rutter, 1988). Some autistic children develop seizures, commonly around the time of puberty.

The cause of autism is not known. However, this syndrome is generally believed to result from several different biologic events or a combination of etiological factors.

Research on genetic, viral, and immunological factors began in the middle 1960's.

*Genetic Implications and Autism:* In 1967 Rutter, Coppen, and Walk determined that the 2% rate of autism in siblings of autistic patients was 50 times that of the general population. Folstein and Rutter (1977) also reported that a family history of speech delay was found in about a quarter of families with autistic children. Thus, they hypothesized that delayed speech in autism is probably caused by a genetic predisposition in the part of the brain responsible for language.

Since autism is found four times more often in males as in females, there is a possible correlation between sex-linkage and autism. Mariner, Jackson, Levitas, and Hagerman (1986) studied chromosomes from autistic individuals and found sex chromosomal abnormalities including XXY, XYY, and fragile X karyotypes. However, structural autosomal defects have not been reported. Another study from Folstein and Rutter (1988) suggests that it is not autism itself that is inherited but rather some genetic abnormalities, like language and sociability, which interact with other factors to produce autism.

Recently, Warren *et al.* (1991, 1992) described evidence for a temporal link between an abnormality in a complement gene and autism. Autistic subjects and their mothers had increased phenotypic frequencies of the C4B null allele (58%

in both the autistic subjects and mothers versus 27% in controls). Homozygosity for this allele is associated with reduced C4 serum levels and may affect autistic patients, especially in concert with a viral infection.

*Viral Infection and Autism:* In 1971, Chess studied 243 preschool children with congenital rubella. Among these children, 10 were identified as having autism. An additional 8 children have had some of the symptoms of autism.

Further study on rubella-associated autism was done by Stubbs (1976). He observed that some autistic children who had previously been given vaccinations against rubella did not produce detectable antibody titers when challenged with the same viral vaccine. This failure of anti-viral antibody production indicates that these autistic children may have had an altered immune response.

In a later study Stubbs (1978) showed an association of congenital cytomegalovirus (CMV) infection with autism. Later, additional cases of CMV infection in autism were reported (Markowitz, 1983; Stubbs, Ash, & Williams, 1984; Ivarsson, Bjerre, Vegfors, & Ahlfors, 1990). Urine samples from these autistic children tested positive for CMV. There were no significant differences between antibody titers for CMV of autistic and control children. However, an interesting discrepancy in the number of children with anti-CMV antibody titers was described (Stubbs, 1987). Only 7% of autistic boys had titers as compared to 75% with titers in autistic girls.

The lower CMV antibody titer in autistic boys indicates that they may be more susceptible to this infection. In Stubbs' 1987 paper, he hypothesized that this susceptibility may explain the 4:1 ratio of boys to girls in autism.

A putative autoantibody produced by an aberrant immune response may attack the areas which are associated with auditory, visual, and motor functions in the brain (Stubbs, 1987). The congenital infections of both rubella and CMV have been suggested to increase the incidence of autism. As the central nervous system is not fully developed at birth, and owing to the immaturity of the brain, even after birth, fetuses and infants are at special risk for the viral damage to the central nervous system. It seems that when autism is associated with congenital virus infection, other disabilities are also present, indicating widespread early damage of the brain (Ivarsson et al., 1990).

*Immunology and Autism:* Several studies have revealed evidence of abnormal immunological regulation in autistic patients. Autistic patients have defective antibody production after vaccination with rubella (Stubbs, 1976). Depressed lymphocyte responsiveness to the T cell mitogen PHA was found in autistic cases (Stubbs et al., 1977).

Weizman, Weizman, Szekely, Wijsenbeek, and Livni (1982) used macrophage migration inhibition factor (MMIF) as a tool and detected abnormality of a cell-mediated immune response to brain myelin basic protein in autistic children. In

association with the autoimmune theory of the etiology of autism, Todd and Ciaranello (1985) reported blocking antibodies against serotonin binding proteins of the rat brain homogenate.

Further study from Warren *et al.* (1986) confirmed the earlier report of Stubbs *et al.* (1977) of the mitogen-induced lymphocyte blastogenesis in autism. Their study indicated that lymphocytes of autistic children also had significantly low responses to the T cell mitogen concanavalin A (ConA) and the B cell mitogen pokeweed mitogen (PWM). However, these mitogenic responses were found to be depressed in only about 45% of autistic patients. In some cases, the response was even higher than the response found in sibling controls as reported by Singh *et al.* (1988). In addition to studying mitogen responses, Warren and associates (1986) also reported the reduced number of total T cells, especially the CD4+ helper T cells, in autistic patients.

Warren, Foster, and Margaretten (1987) found that approximately 40% of autistic children had reduced natural killer (NK) cell activity. This altered NK activity could be related to risk factors such as viruses. In addition, autistic patients have an increased incidence of circulating antibodies that are directed against neuron axon filament proteins (Singh *et al.*, 1988).

In recent immunophenotyping studies with monoclonal antibodies and fluorescence-activated cell sorter (FACS)

analysis, there were significantly reduced proportions of total lymphocytes, CD2+, CD4+, and CD4+CD45RA+ T cells in the peripheral blood of patients with autism (Warren et al., 1990; Yonk et al., 1990).

Thus, all of the aforementioned immunological abnormalities point to an immunopathogenetic mechanism, perhaps autoimmunity, as one important causation in autism.

#### *Alzheimer's Disease*

Alzheimer's Disease (AD) is associated with degenerative changes throughout the brain, particularly in the basal forebrain region which provides most of the cholinergic innervation of the cortex and hippocampus. Patients with AD are generally characterized by progressive loss of memory and intellectual function, delusional or paranoid features, and activity disturbances. Because the majority of AD patients are over 60 years of age, AD has become most noticeable among the elderly in our society. The disease is now considered as the fourth leading killer of adults, taking more than 100,000 lives annually (Singh, White, Yonk, Warren, Burger, Karen, & Singh, 1990).

Diagnosing AD requires precise clinical and laboratory methods. Neurological techniques, including magnetic resonance imaging (MRI), positron emission tomography (PET), and electroencephalogram (EEG) scans, are used as tools to study the disease. Since a systematic progression of the

development of symptoms in AD is related to the decline of memory function, neuropsychiatric assessment is used to evaluate AD patients. The mini-mental-state examination (MMSE) is a useful clinical method for evaluating the disease (Folstein, Folstein, & McHugh, 1975). Data from MMSE are analyzed by item characteristic curve analysis (ICC), which can help to delineate the loss of mental functions during the course of AD (Ashford, Kolm, Colliver, Bekian, & Hsu, 1989).

The actual cause of AD is not known. Different approaches with biochemistry or immunology are used.

*Biochemical Abnormalities in AD Brains:* Biochemical studies of AD brain tissue reveal several abnormalities. One is a marked deficiency in the activity of choline acetyltransferase (ChAT), the enzyme responsible for the synthesis of the neurotransmitter acetylcholine (ACh). Acetylcholine is a chemical involved in human memory function.

Another finding was a greater cognitive impairment associated with lower corticotropin-releasing factor (CRF) concentrations in Alzheimer's patients than in normal controls (Singh & Fudenberg, 1988a; Pomara et al., 1989). Studies from Pomara et al. (1989) also described a significant reduction in the cerebral spinal fluid (CSF) concentration of homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) in the Alzheimer's patients. A putative link between these abnormal biochemical findings and the immune system is difficult to

draw clearly. However, other biochemical studies have been more conclusive, especially for CRF.

The concentration of a cytoplasmic brain protein of 68 kDa as detected by a monoclonal antibody (designated as Alz-50) is markedly increased in an Alzheimer's patient as compared to normal controls (Wolozin, Pruchnicki, Dickson, & Davies, 1986). In another study the alpha 1-antichymotrypsin (alpha 1-ACT) was increased significantly in the serum and CSF of AD patients, which suggested that alpha 1-ACT can be used as an antemortem biological marker of AD (Hirai, 1990). Singh (1990) has recently discussed CNS-Immune system interactions through neuropeptides and neurotransmitters, including endorphins, corticotropins, acetylcholine, serotonin, dopamine, histamine, substance P, somatostatin, and corticotropin-releasing factor (CRF).

Some neurochemical changes in AD brains correlate with the deposition of neurofibrillary tangles (NFT) and the formation of neuritic plaques (NP). The NFTs are twisted pairs of fine nerve fibers, composed of paired helical filaments (PHF), which accumulate in the cell bodies of neurons whereas the NPs consist of clusters of neurites, enlarged nerve endings, and unmyelinated axons around a core of fibrous material called "amyloid" (Singh & Fudenberg, 1988b). The exact biochemical nature of NFT and NP is not known. Antiserum to neurofilaments of intermediate size was immunoreactive with NFT (Yen, Gaskin, & Fu, 1983; Gambetti,

Gambetti, Perry, Schecket, & Cane, 1983). In addition, the presence of ubiquitin protein was detected by sequence analysis in PHF (Mori, Kondo, & Ihara, 1987).

*Immunology and AD:* Although the etiology and pathogenesis of AD are not known, in several reports the involvement of immunological mechanisms was hypothesized (Singh & Fudenberg, 1986). Abnormal functions of cell-mediated and humoral immunity were demonstrated in AD patients. In regard to cell-mediated immunity, lymphocyte proliferation in response to mitogens (PHA, ConA, PWM, and monoclonal antibody to CD3 antigen of T cells) was significantly decreased in AD patients as compared to age-matched controls (Singh, Fudenberg, & Brown, 1987). The mean proliferative response of T cells in an autologous mixed lymphocyte reaction (AMLR) was increased in a subset of patients (Leonardi *et al.*, 1989). In recent findings the ratio of helper/suppressor T cells was increased in many AD patients (Singh, 1990). These data can be interpreted as an immunoregulatory defect of T cells in AD patients.

Impairment of antibody-mediated immunity also occurs in many patients with AD. Serum levels of immunoglobulins (IgG, IgM, and IgA) were changed invariably (Cohen *et al.*, 1986). In another study there was an increase in the level of IgG3 in 45% of AD patients, while the distribution of IgG1, IgG2, and IgG4 was normal (Singh & Fudenberg, 1989). In addition, nearly 60% of AD patients have serum autoantibodies to brain

tissue antigens (Singh & Fudenberg, 1986). They appeared to be of IgG3 subclass (Singh & Fudenberg, 1989). When the immune complexes containing IgG antibody deposit in tissue, they can cause tissue damage in a classical complement pathway. McGeer, Akiyama, Itagaki, and McGeer (1989) suggested two possible ways that the immune system might be involved in AD. One of these is by the attachment of complement proteins to damaged tissues. IgG3 subclass is the main isotype of brain autoantibody in AD (Singh & Fudenberg, 1989). It can effectively activate the classical pathway of complement system and cause tissue damage. Immunohistochemical staining of AD brain tissue was obtained with antibodies to proteins associated with the classical, but not the alternative, pathway (McGeer *et al.*, 1989).

The other possible suggested mechanism would be by the activation of cells associated with the immune system (Singh & Fudenberg, 1986). The leucocyte common antigen (LCA) positive cells, including both helper and suppressor T cells, were identified in the brain tissue of AD patients (Itagaki, McGeer, & Akiyama, 1988). Other immunity-related antigens, including IL-1, IL-2R, HLA-DR, and CD4+, were also found in AD brain tissue (Rogers & Mufson, 1990; Ferrar, Kilian, Ruff, Hill, & Pert, 1987; Funke, Hahn, Rieber, Weiss, & Reithmuller, 1987). IgG from sera of AD patients binds to neurofilament subunit (NF-H) in torpedo cholinergic neurons more than control IgG (Michaelson, Chapman, Bachar, Korczyn, & Wertman,

1989). In 1990, Blennow and his co-workers found that AD patients displayed an intra-blood-brain-barrier (intra-BBB) for immunoglobulin (IgG and IgM) synthesis, but none was seen among the controls.

Based on the studies of interactions between the central nervous system and the immune system, it was recently hypothesized (Singh, 1990) that the neuroimmune axis may be one possible approach to therapy in AD. The acetylcholinesterase enzyme activity of blood lymphocytes is lower in AD patients than in aged controls (Bartha et al., 1987). The mitogen-induced uptake of calcium by blood lymphocytes is decreased in AD patients (Gibson, Nielson, Sherman, & Blass, 1987). The membrane fluidity of blood platelets is reduced in AD patients. Nearly 80% of AD patients showed markedly reduced binding of corticotropin-releasing factor (CRF) of neurohormone to the peripheral blood lymphocytes (Singh & Fudenberg, 1988a). The blood lymphocytes from AD patients displayed significantly lower binding of [<sup>3</sup>H]-serotonin neurotransmitter when compared to the aged controls (Singh, Warren, & Singh, 1990).

Thus, from the studies in the two subheadings above it is clear that biochemical research has supported the significance of immunological abnormalities in AD. As was discussed in the section on autism research, AD research also points to an immunopathogenetic mechanism, perhaps autoimmunity, as one important causation in AD. Putative CNS-immune system links

have been made by studying the correlation between biochemical abnormalities in the brains of autistic and AD patients and the causes or effects of these abnormalities by or on the immune system.

*Human Thymocytes*

The thymus, one of the primary lymphoid organs, is present in the thorax and is the site of ontogenetic development of T lymphocytes. The cell populations in the thymus include thymocytes, macrophages, dendritic cells, and thymic epithelial cells. In humans, 3 stages of intrathymic maturation of T cells are defined based on the expression of cluster differentiation phenotypic specific antigens on the T cell surface (Sanchez-Madrid, Toribio, Gambon, & De Landazuri, 1985).

During the first stage, the earliest identifiable T cell lineage displays CD2+ (the sheep erythrocyte binding glycoprotein) molecules. At this stage, no other T cell-specific molecules are expressed. The second stage is defined by the co-expression of CD3, CD4, and CD8 molecules. Thymocytes in these 2 stages are mainly located in the thymic cortex and represent about 85% of the total thymic lymphocyte population.

During the last stage, which is in the thymic medulla, the T cell lineage splits into 2 parallel lines, both lines maintaining the CD3 molecule. One line is CD4+ and CD8- while

the other is CD4- and CD8+. These 2 populations of T lymphocytes then enter the secondary lymphoid tissues. The CD2+, CD3+, and CD4+ cells (helper T cells) represent about 65% of peripheral blood T cells, and the CD2+, CD3+, and CD8+ cells (suppressor T cells) represent about 35% of peripheral blood T cells.

#### *Murine Thymocytes*

In a normal mouse, the stem cells enter the thymus at day 11 to 12 of gestation. They stay in the thymus for maturation and thymic selection. The surface antigens, Thy-1, Pgp-1 (Ly-24), and IL-2R, are found on thymocytes at day 13 during development. The Thy-1 antigen is present on the membrane thereafter, but Pgp-1 and IL-2R are found mostly on early thymocytes and are down regulated toward the end of gestation. At about day 16, CD4, and CD8 molecules appear and are highly expressed on day-18 thymocytes. Gene rearrangement of T cell receptor (TCR) starts at about day 12 to 13. After birth, the alpha and beta chains of TCR are found on the majority of thymocytes and peripheral T lymphocytes. The gamma and delta chains are highly expressed at early stages of thymocyte development, but only appear on a minority of T cells (Berger & Epstein, 1989).

*Thymic Epithelial Cells and Thymocytes*

Thymic epithelial cells are found as regular and substantial components in the thymus. These cells have an unusually large size (diameters of 30  $\mu$ l and more) and are termed thymic nurse cells (TNC). When thymocytes contact these epithelial cells, they are completely surrounded by the epithelial cell membranes. At this time many of thymocytes undergo mitosis.

Evidence (Wekerle, Ketelsen, & Ernst, 1980) suggests that structures which morphologically resemble certain stages of gap junctions are formed between thymocyte and epithelial cell membranes. Thus, nutrition, as well as signal substances, can be exchanged. Thymic epithelial cells can secrete some thymic hormones (thymosin, thymopoietin, etc.) which can induce thymic cell differentiation and maturation.

Thymic epithelial cells express a high dose of major histocompatibility complex (MHC) antigen molecules (both class I and class II). Only those thymocytes that are able to recognize and interact with these MHC determinants can survive. Other cells with inappropriate reactivities will die.

Both humoral secretion and MHC interaction of thymic epithelial cells are important. When either one of these two functions is aberrant, an immunological abnormality may occur.

*The Complement System*

There are at least 20 chemically and immunologically distinct plasma proteins in the complement system. These proteins are present in the circulation and are usually functionally inactive. Once they are activated by foreign molecules or antibodies, they are capable of interacting with each other in the fashion analogous to substrate-enzyme reactions. Together, they make up approximately 15% of plasma globular proteins. After heating at 56°C for 30 minutes, the biological activity of many of these proteins is destroyed (Cooper, 1987).

In the complement system, different agents can induce different but parallel pathways which cause cell lysis. The two major mechanisms are the classical pathway and the alternative pathway. The classical pathway can be activated by antigen-antibody complexes with human immunoglobulins (IgG or IgM). For instance, the first complement component (C1) can use the C1q subcomponent triple helix structures to bind to the C<sub>H</sub>2 domains of IgG molecules and trigger the classical pathway. IgG1, IgG2, and IgG3 subclasses are the main immunoglobulins in the IgG subclasses that are involved in the induction of classical pathway (Cooper, 1987).

Activation of alternative pathway proceeds in a different pathway from that of the classical pathway and does not require specific antibody. Insoluble yeast cell wall preparation (zymosan), and plant and bacterial polysaccharides

are activators for the alternative pathway. Activated alternative pathway enzymes assemble themselves on the target cell membrane and cleave a C3b fragment from C3. Factor B and C3b combine to form C3b<sub>n</sub>Bb. This form is then cleaved by factor D and C3b<sub>n</sub>Bb (C3 convertase) is formed. Properdin (P) can stabilize C3 convertase by binding to C3b<sub>n</sub>Bb and forming C3b<sub>n</sub>PBb (Cooper, 1987).

After a series of cleavage reactions, both pathways generate a C3 convertase and converge at the midpoint of the complement system. The C3 convertases of both pathways then bind further C3b to yield the C5 convertases (C4b<sub>2</sub>a<sub>3</sub>b in classical pathway, and C3b<sub>n</sub>PBb in alternative pathway) which activate the next component of the complement system, C5. The C5 convertases split C5 into fragments of C5a and C5b. The fixation of C5b to biological membranes is followed by the sequential addition of four more proteins C6, C7, C8, and C9. The MC5b<sub>6,7,8,9</sub> complex, known as the membrane attack complex, is formed and inserted into the cell membrane and causes cell lysis (Cooper, 1987).

#### MATERIALS AND METHODS

##### *Statistical Methods*

Data from the assays were analyzed using a two-way ANOVA test. Significance was set at P < 0.05. Error bars on the graphs of proliferation response and cytotoxic activity were representative of the standard error of the mean (SEM).

*Experimental Subjects*

The subjects used in this study included white autistic patients from Northern Utah, AD patients, and age-matched healthy controls. The age of autistic patients ranged from 4 to 33 years with a mean age of 13.7 years. All of the patients satisfied the revised third edition of the Diagnostic and Statistical Manual (DSM-III-R) criteria for the diagnosis of autism and had been under the care of a physician since the original diagnosis of autism. Twenty-one healthy controls were randomly selected to match as far as possible the age of the patients. The mean age of the control group was 16.3 years in the range of 4 to 36 years.

In the Alzheimer's study, the mean age of 9 patients was 83 years, ranging from 59 to 96 years. The clinical diagnosis of AD was made by a neurologist based on the DSM-III-R criteria of exclusion of all other reasons of dementia but Alzheimer's disease. Eleven age-matched controls, with a mean age of 73.5 years, ranging from 53 to 90 years, were compared to the patient's group.

*Collection of Human Plasma Samples*

After appropriate consent forms were signed, approximately 60 ml of peripheral blood were drawn with a syringe containing about 0.6 ml of preservative-free heparin (Sigma, St. Louis, MO, 0.1 ml heparin, 100-250 Units, for 10 ml blood). Plasma was removed following centrifugation at 500

g for 10 to 15 minutes and was stored frozen at -70°C until further study. Heparin does not interfere with antigen-antibody complex formation. The only effect would have to be caused by the clotting factors found in plasma that are not found in serum. Clotting factors have not been indicated as important in studying autism or AD and should not affect the stability of the antigen-antibody complexes formed.

*Collection and Preparation of Murine Thymocytes*

Thymuses from 3 to 6 black mice (USU LARC, C57BL/6 strain, male or female) were dissected each time. The thymuses were kept in a 15 ml test tube (Corning, New York, NY) with 10 ml RPMI-1640 (Gibco, Grand Island, NY). A 5 cc syringe was used to mash the thymuses, and then thymocytes were collected. The cell suspension was pipetted into a 15 ml test tube (Corning) and centrifuged at 600 g for 5 minutes. After the centrifugation, 9 ml of double-distilled water were added for 10 seconds to lyse red blood cells. One ml of 10 x phosphate buffered saline (PBS, containing 0.01 M sodium phosphate, 0.154 M sodium chloride, pH 7.4) was used to stop the reaction.

Wood applicators were used to remove the debris. The samples were centrifuged at 600 g and washed 2 more times with RPMI-1640 medium (Gibco). A small volume of the cell suspension was diluted with crystal violet solution, and the thymocytes were counted on a hematocytometer. Cell

concentrations (described in the methods below) were adjusted for ELISA, proliferation response of thymic cells assay, and cytotoxicity assay.

#### *Detection of Antithymic Antibodies*

The basis of this method was to immobilize thymic cells onto the polystyrene surface of a microplate, using the details of a microplate Raji cell ELISA method as described by Singh and Kwong (1984). The murine thymocytes were collected from fresh thymuses and washed 3 times with PBS buffer. After the final wash, the cells were resuspended in PBS and adjusted to a concentration of  $1 \times 10^7$  cells/ml. Two hundred  $\mu\text{l}$  of the cell suspension were pipetted and distributed evenly into each well of a 96-well, flat-bottomed microplate with polystyrene surface (Corning).

The cells were allowed to settle to the bottom of the well by incubating the plate at room temperature for at least 1 hour. After settling, 25  $\mu\text{l}/\text{well}$  of 2% (w/v) glutaraldehyde solution were carefully pipetted into each well without disturbing the cell layer. The plate was put into a 4°C refrigerator for 4 hours for immobilization of cells to the plastic surface. After immobilization, the plate was washed 10 times with PBS to completely remove the glutaraldehyde.

One hundred  $\mu\text{l}$  of the 2-fold diluted (1/2.5, 1/5, 1/10, 1/20) normal and patient plasma (as the source of first antibody) were added in duplicated wells; 0.1 ml PBS was used

to set up background binding. The plate was incubated for 30 minutes at 37°C in a 5% CO<sub>2</sub>-incubator with 85% humidity and was then washed 4 times with PBS. One hundred  $\mu$ l of 1:1000 diluted goat-anti-human IgG antibody conjugated with alkaline phosphatase (Sigma, St. Louis, MO) were added to each well and the plate was incubated for another 30 minutes.

Following this incubation, the excess enzyme conjugate was decanted and the plate washed 4 times with PBS. One hundred  $\mu$ l of a freshly prepared 1 mg/ml solution of p-nitrophenylphosphate (5 mg tablets from Sigma) in 0.05 M Na<sub>2</sub>CO<sub>3</sub> buffer, pH 9.6, containing 1 mM MgCl<sub>2</sub>, were pipetted into each well. The plate was maintained at room temperature for 30 minutes. The enzyme reaction was terminated by the addition of 25  $\mu$ l/well of 1 N NaOH solution. The absorbance was read at 405 nm using a Microplate Reader (Bio-Rad Model 450).

#### *Proliferation of Thymic Cells*

This assay was done by measuring [<sup>3</sup>H]-methyl-thymidine uptake by thymocytes incubated in the presence or absence of normal human plasma, or patient plasma. Two-fold dilutions (40%, 20%, 10%, 5%, 2.5%, 1.25%) of each plasma sample in RPMI-1640 (Gibco) were tested in triplicate cultures, which contained 50  $\mu$ l of thymocytes ( $2 \times 10^6$  cells/ml), 50  $\mu$ l of rIL-1 (2.5 unit/ml, Cistron, Pine Brook, NJ), 50  $\mu$ l of a 4% stock solution of PHA (Gibco), and 50  $\mu$ l of diluted plasma.

The plates were incubated at 37°C for 3 days. After the incubation, 0.5  $\mu$ Ci of [ $^3$ H]-methyl-thymidine with a specific activity of 2 Ci/mmol (DuPont-New England Nuclear, Boston, MA) was added to each well. The plates were incubated overnight. After the final incubation, the cell cultures were harvested on glass fiber filter paper disks using a Micromate 196 Harvester (Packard). This labeling and harvesting method was the same as that described elsewhere (Singh et al., 1987). The discs were placed in scintillation vials, and 2 ml of scintillation cocktail (Ready Safe, Beckman, Irvine, CA) were added to each vial. The radioactivity was counted using a Packard Model-1500 Tri-Carb Liquid Scintillation Analyzer. The counts per minute (CPM) divided by the disintegration per minute (DPM) yielded a 52% counting efficiency. However, the results were calculated using DPM only. Thus, the results should be independent of the counting efficiency.

#### *Assay for Complement-Dependent Cytotoxic Activity*

The basis of this assay method was to measure cell lysis in the presence of specific factors, such as antibodies, or non-specific factors, such as complements. The cell cytotoxicity was measured by  $^{51}$ Cr release assay as described by Warren et al. (1987) for NK cell assay.

The target cells, murine thymocytes, were adjusted at 3 to  $5 \times 10^6$  cells/ml in RPMI-1640 (Gibco) and centrifuged at 500 g for 5 minutes. The supernatant was decanted and 0.1 ml of

sodium chromate ( $\text{Na}_2^{51}\text{CrO}_4$ ) containing 1  $\mu\text{ci}/\text{ml}$  was added to the cell pellet, and incubated for 2 hours at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  incubator.

Normal and patient plasmas were pipetted into duplicate sets of 2 ml plastic test tubes. One set was heated in a  $56^\circ\text{C}$  waterbath for 30 minutes; the other one was kept at room temperature. After 30 minutes, the inactivated plasma was split into 2 tubes, and 2-fold dilutions (1/64, 1/128, 1/256, 1/512) were carried out for all 3 tubes of plasma in the 96-well microplate (Corning). A 20  $\mu\text{l}/\text{well}$  aliquot of 1:10 diluted rabbit complement (Pel-Freez, Brown Deer, WI) was added to 1 set of 2-fold diluted inactivated plasma.

After 2 hours of  $^{51}\text{Cr}$ -labelling, target cells were washed 3 times in RPMI-1640 (Gibco). Cells were washed by centrifuging for 5 minutes at 500 g, decanting the supernatant, and adding 30 ml RPMI-1640 (Gibco) containing less FBS (HyClone, Logan, UT) after each resuspension. The first wash contained 20% FBS (HyClone). Ten percent FBS was in the second. No FBS was in RPMI-1640 (Gibco) added during the last wash. FBS-free RPMI-1640 was also used to resuspend the final pellet at a concentration of  $1 \times 10^5$  cells/ml. One hundred  $\mu\text{l}$  of the labelled target cells were then added to each well. The plate was incubated at  $37^\circ\text{C}$  for 4 hours, centrifuged at 200 g for 5 minutes, and an aliquot (0.1 ml) of supernatant was carefully harvested from each well and placed in a vial (Sarstedt, Pennsauken, NJ) and counted in a gamma

counter (Packard Gamma Scintillation Spectrometer). The percentage of cell cytotoxicity, as measured in terms of the  $^{51}\text{Cr}$  release, was calculated by the following formula:

$$\% \text{ cytotoxicity} = \frac{\text{experimental cpm} - \text{background cpm}}{\text{maximal cpm} - \text{background cpm}} \times 100$$

The experimental release (cpm) was the result of the lysis of thymocytes during incubation with plasma, the maximum release (cpm) was determined by 5% saponin treatment of an aliquot of target cells, and the background release (cpm) was obtained by incubating an aliquot of thymocytes in growth medium alone.

## RESULTS

### *Detection of Antithymic Antibodies*

The results of studying antithymic antibodies in the plasmas of autistic subjects are presented in Figure 1. The mean absorbance values obtained from these plasmas in ELISA were significantly ( $P < 0.05$ ) increased, in a dilution-related fashion, as compared to values obtained from plasmas of age-matched control subjects. The results of investigating antithymic antibodies in plasmas of the Alzheimer's patients are given in Figure 2. The results of studying antithymic antibodies in the plasmas of individual autistic and Alzheimer's subjects are given in Figures 3-6.

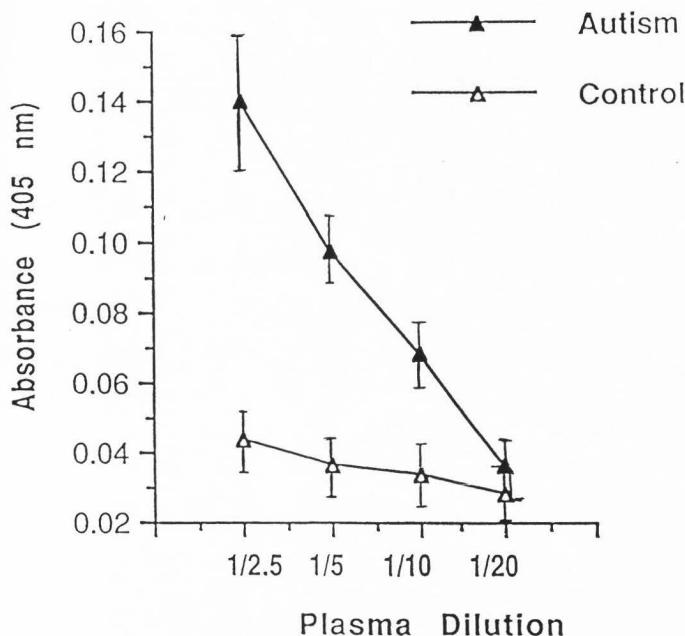


Figure 1 Antithymic antibodies in autistic patients.

The mean absorbance values, as assessed by ELISA for antithymic antibodies, were calculated when using plasma samples of 16 autistic patients and compared to those calculated when using plasma samples of 8 normal control subjects,  $P < 0.05$ . Error bars on the graphs represent the standard error of the mean (SEM).

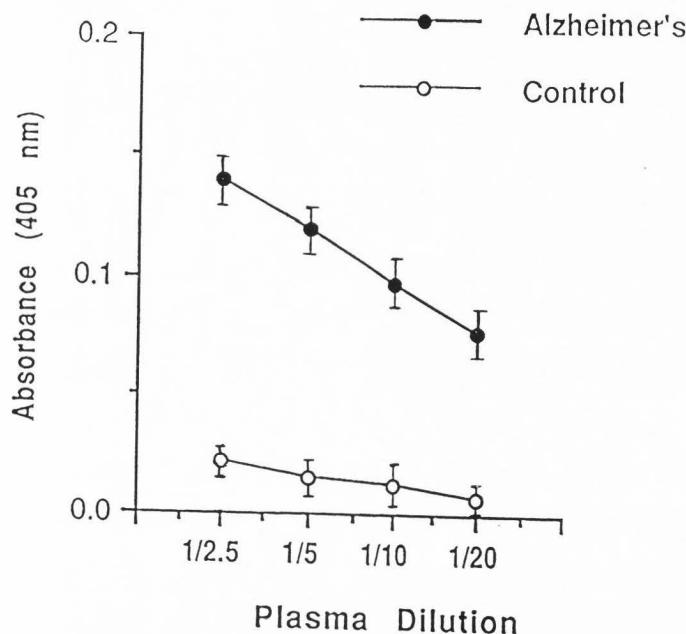


Figure 2 Antithymic antibodies in AD patients.

The mean absorbance values, as assessed by ELISA, were calculated when using plasma samples from 8 AD patients and compared to those calculated when using plasma samples of 13 normal control subjects,  $P < 0.001$ . Error bars on the graphs represent the standard error of the mean (SEM).

## 1/2.5 Dilution

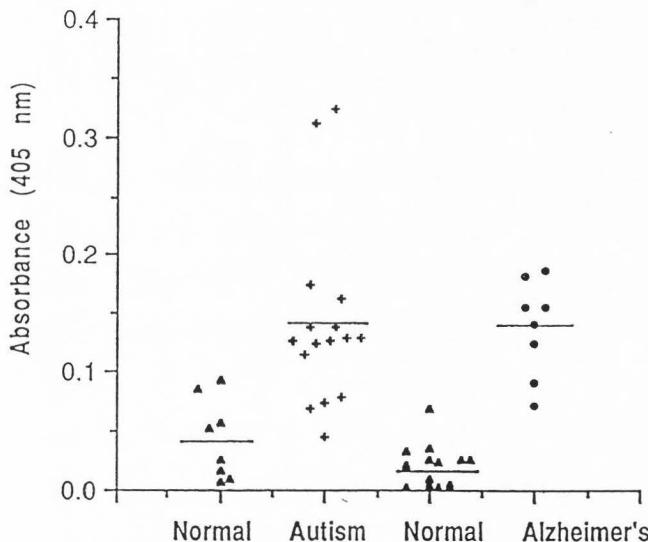


Figure 3 ELISA data for plasmas diluted 1:2.5.

Individual absorbance values are shown for 4 different groups. The mean value for the controls age-matched to the autistic group ( $\blacktriangle$ ) was 0.044 ( $N = 8$ ). For the autistic patients ( $\times$ ) it was 0.14 ( $N = 16$ ). For the controls age-matched to the Alzheimer's group ( $\blacktriangle$ ) it was 0.022 ( $N = 13$ ). For the Alzheimer's patients ( $\bullet$ ) it was 0.14 ( $N = 8$ ).

## 1/5 Dilution

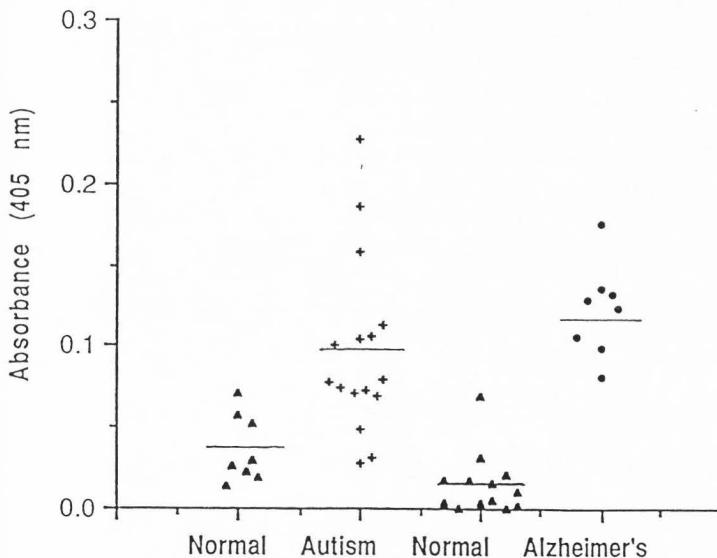


Figure 4 ELISA data for plasmas diluted 1:5.

Individual absorbance values are shown for 4 different groups. The mean value for the controls age-matched to the autistic group ( $\blacktriangle$ ) was 0.036 ( $N = 8$ ). For the autistic patients (+) it was 0.097 ( $N = 16$ ). For the controls age-matched to the Alzheimer's group ( $\blacktriangle$ ) it was 0.015 ( $N = 13$ ). For the Alzheimer's patients (●) it was 0.12 ( $N = 8$ ).

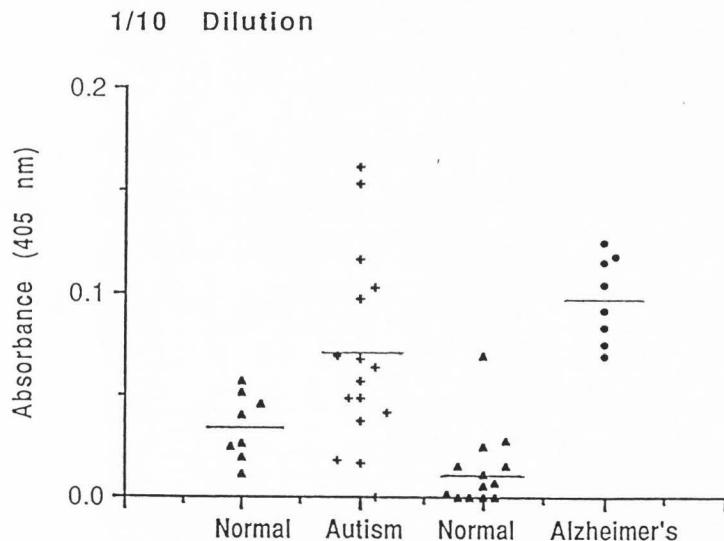


Figure 5 ELISA data for plasmas diluted 1:10.

Individual absorbance values are shown for 4 different groups. The mean value for the controls age-matched to the autistic group (▲) was 0.034 (N = 8). For the autistic patients (+) it was 0.068 (N = 16). For the controls age-matched to the Alzheimer's group (▲) it was 0.012 (N = 13). For the Alzheimer's patients (●) it was 0.097 (N = 8).

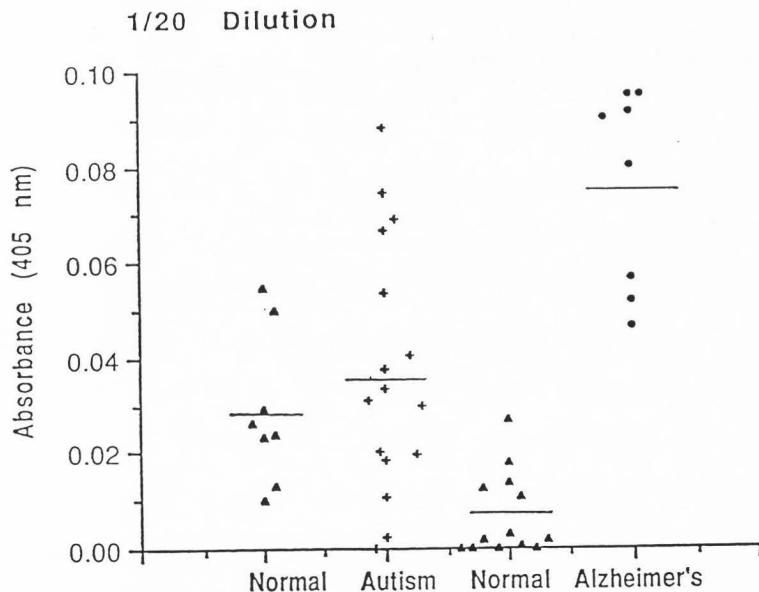


Figure 6 ELISA data for plasmas diluted 1:20.

Individual absorbance values are shown for 4 different groups. The mean value for the controls age-matched to the autistic group ( $\blacktriangle$ ) was 0.028 ( $N = 8$ ). For the autistic patients ( $\ast$ ) it was 0.036 ( $N = 16$ ). For the controls age-matched to the Alzheimer's group ( $\blacktriangle$ ) it was 0.0063 ( $N = 13$ ). For the Alzheimer's patients ( $\bullet$ ) it was 0.076 ( $N = 8$ ).

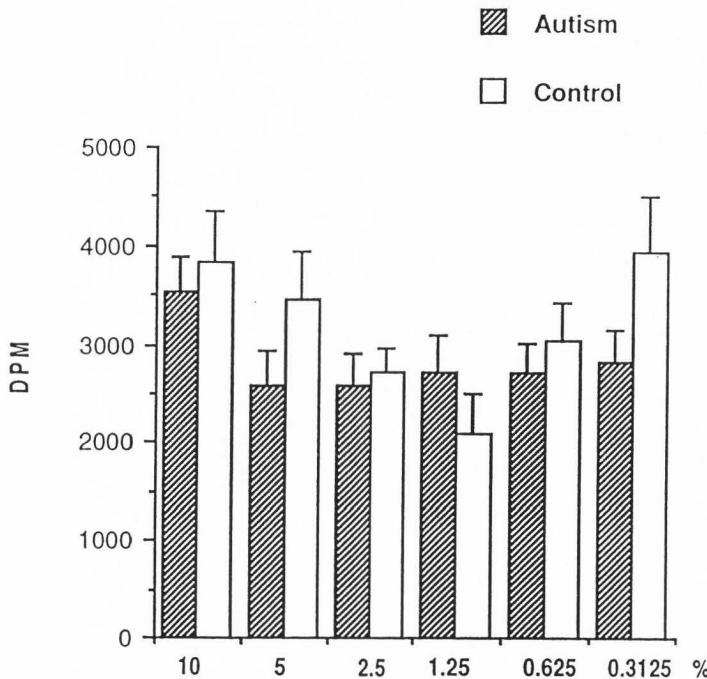


Figure 7 Proliferation using autistic patient plasmas.

The means of thymic-cell uptakes of [ $^3\text{H}$ ]-methyl-thymidine, as expressed in disintegrations per minute (DPM), were compared between cells incubated in autistic patient plasmas ( $N = 16$ ) and plasmas of controls ( $N = 8$ ). Error bars on the graphs represent the standard error of the mean (SEM). No significant differences were found.

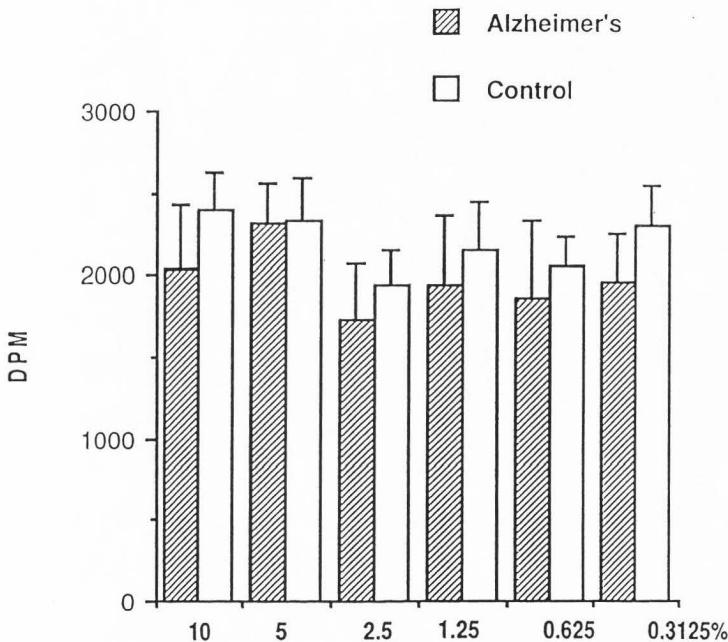


Figure 8 Proliferation using AD patient plasmas.

The means of thymic-cell uptakes of  $[^3\text{H}]$ -methylthymidine, as expressed in disintegrations per minute (DPM), were compared between cells incubated in Alzheimer's patient plasmas ( $N = 6$ ) and plasmas of matched controls ( $N = 10$ ). Error bars on the graphs represent the standard error of the mean (SEM). No significant differences were found.

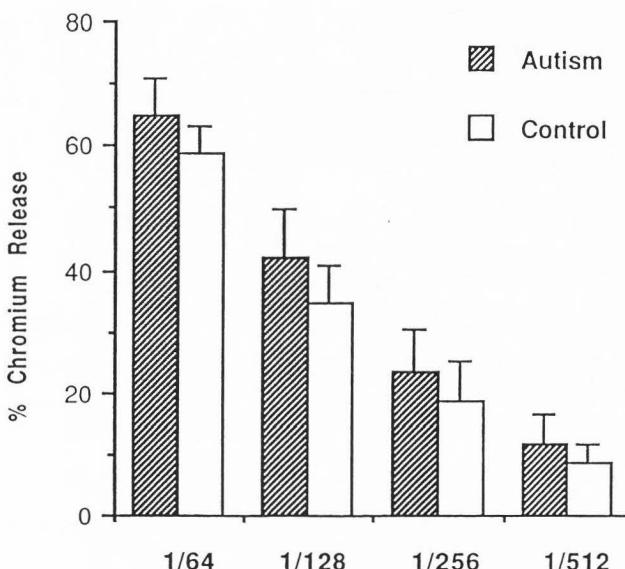


Figure 9 Untreated autistic patient plasmas.

The means of cytotoxic activities, expressed as a percentage of chromium released, were compared between cells incubated in untreated autistic patient plasmas ( $N = 14$ ) and untreated plasmas of matched controls ( $N = 13$ ). Error bars on the graphs represent the standard error of the mean (SEM). No significant differences were found.

decreased levels of chromium release from the thymus cells (Figure 10). However, no significant differences in releases were effected by treated patient plasmas as compared to control plasmas. Addition of rabbit complement to the incubation of treated plasmas and thymic cells resulted in increased chromium release (Figure 11) with a statistically significant ( $P < 0.05$ ) increase at the 1/64 and 1/128 plasma dilutions of the patient plasmas as compared to the controls.

*Alzheimer's Patient Plasma:* Figure 12 is a graph of the percentage of chromium release from thymus cells incubated with untreated patient and control plasmas. No statistically significant differences were seen. When thymus cells were incubated with heat-treated plasma, as presented in Figure 13, the plasmas were less cytotoxic to the thymus cells with no significant difference seen between samples from Alzheimer's and control subjects. Figure 14 represents the results after adding the rabbit complement to the assays. No significant difference in results was seen using the patient samples as compared to control samples. The individual results of studying cytotoxicity of patient and control plasmas are given in Figures 15-26.

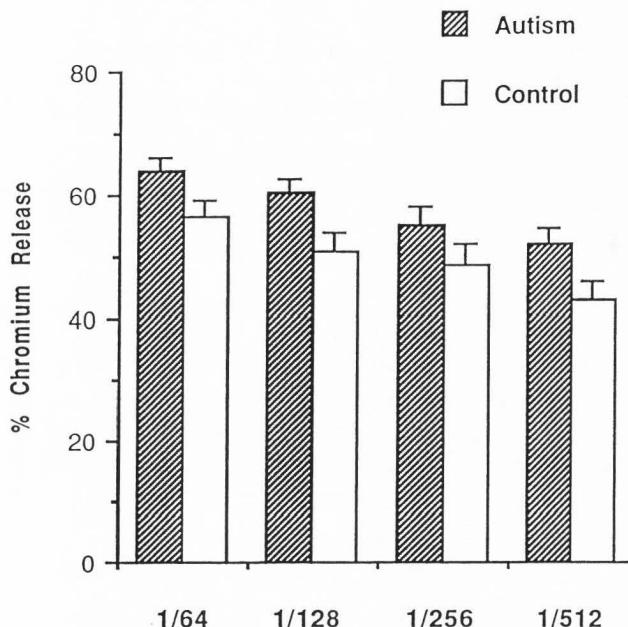


Figure 11 Complement-treated autistic patient plasmas.

The means of cytotoxic activities, expressed as a percentage of chromium released, were compared between cells incubated in 1:10 diluted rabbit complement ( $20\mu\text{l}/\text{well}$ ) added to heat-treated autistic patient plasmas ( $N = 14$ ) and heat-treated plasmas of matched controls ( $N = 13$ ). Error bars on the graphs represent the standard error of the mean (SEM). Significant differences were found only at dilutions of 1/64 ( $P = 0.043$ ) and 1/128 ( $P = 0.013$ ).

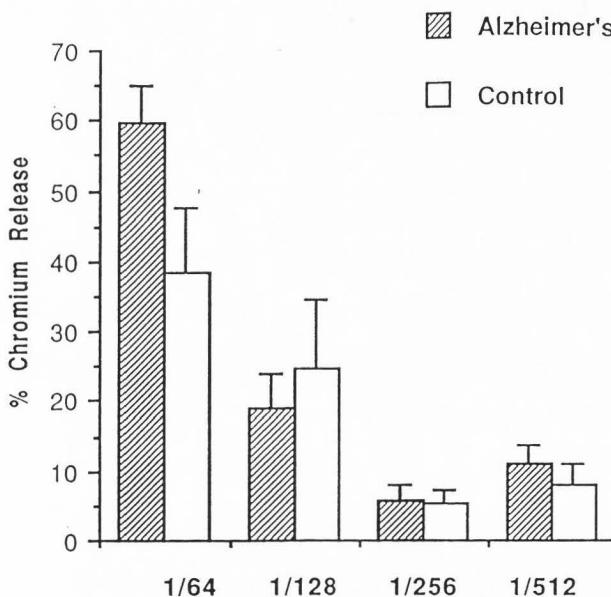


Figure 12 Untreated AD patient plasmas.

The means of cytotoxic activities, expressed as a percentage of chromium released, were compared between cells incubated in untreated autistic patient plasmas ( $N = 6$ ) and untreated plasmas of matched controls ( $N = 7$ ). Error bars on the graphs represent the standard error of the mean (SEM). No significant differences were found.

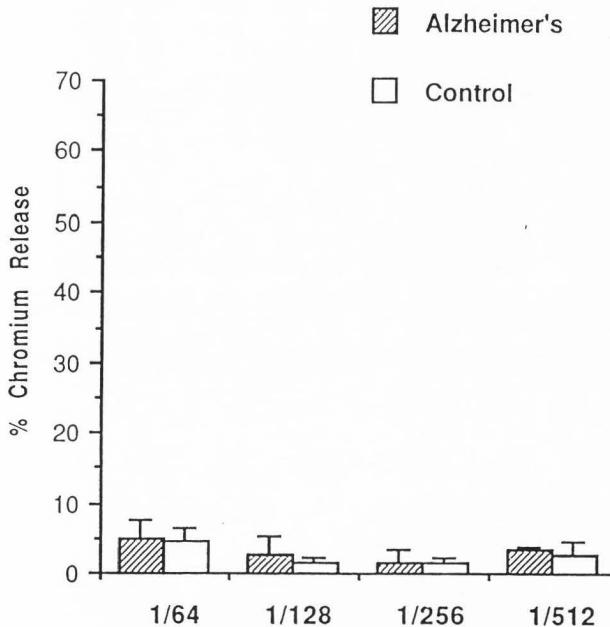


Figure 13 Heat-treated AD patient plasmas.

The means of cytotoxic activities, expressed as a percentage of chromium released, were compared between cells incubated in heat-treated autistic patient plasmas ( $N = 6$ ) and heat-treated plasmas of matched controls ( $N = 7$ ). Error bars on the graphs represent the standard error of the mean (SEM). No significant differences were found.

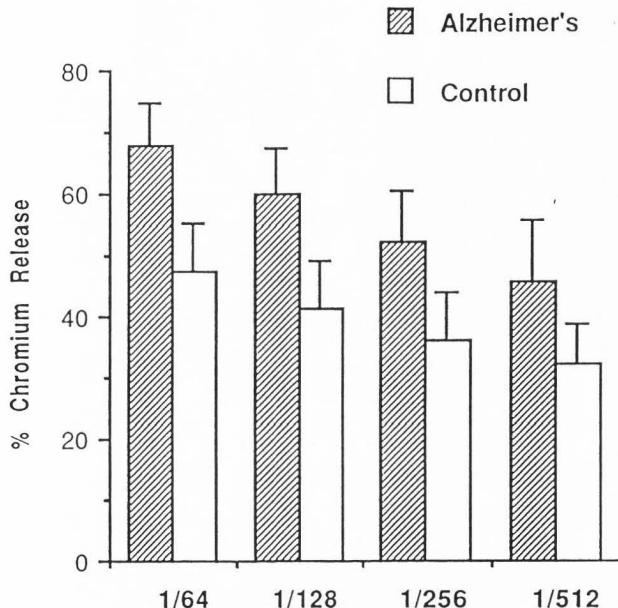


Figure 14 Complement-treated AD patient plasmas.

The means of cytotoxic activities, expressed as a percentage of chromium released, were compared between cells incubated in 1:10 diluted rabbit complement (20 $\mu$ l/well) added to heat-treated autistic patient plasmas ( $N = 6$ ) and heat-treated plasmas of matched controls ( $N = 7$ ). Error bars on the graphs represent the standard error of the mean (SEM). No significant differences were found.

Cytotoxicity of Untreated  
Plasma at a 1/64 Dilution

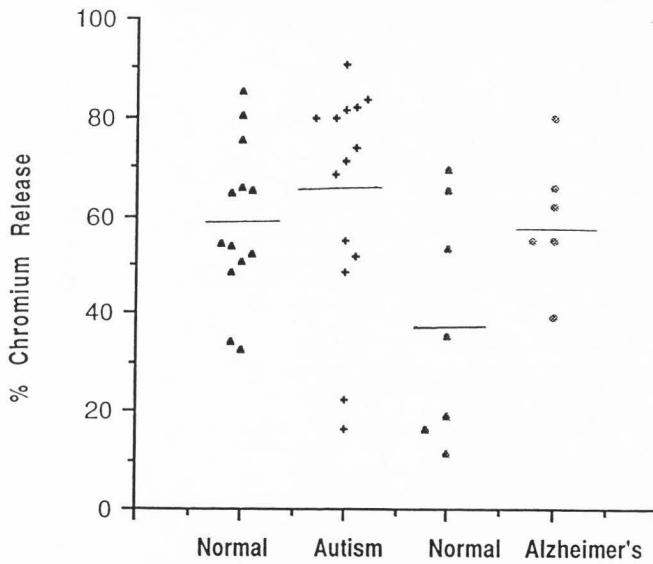


Figure 15 Untreated plasmas diluted 1:64.

Individual cytotoxic activities, expressed as percentages of chromium released, are shown for 4 different groups. The mean value for the controls age-matched to the autistic group (▲) was 58.7% (N = 13). For the autistic patients (✖) it was 64.7% (N = 14). For the controls age-matched to the Alzheimer's group (●) it was 38.5% (N = 7). For the Alzheimer's patients (○) it was 59.6% (N = 6).

Cytotoxicity of Untreated  
Plasma at a 1/128 Dilution

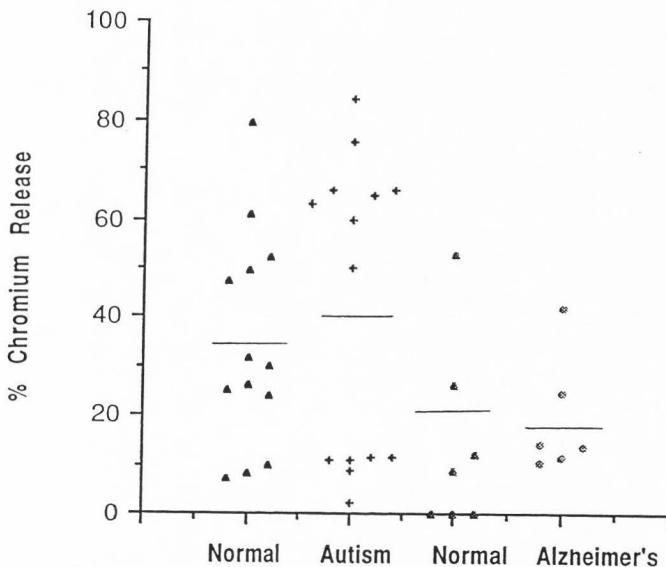


Figure 16 Untreated plasmas diluted 1:128.

Individual cytotoxic activities, expressed as percentages of chromium release, are shown for 4 different groups. The mean value for the controls age-matched to the autistic group (▲) was 34.7% (N = 13). For the autistic patients (+) it was 41.8% (N = 14). For the controls age-matched to the Alzheimer's group (▲) it was 24.9% (N = 7). For the Alzheimer's patients (•) it was 19.2% (N = 6).

Cytotoxicity of Untreated  
Plasma at a 1/256 Dilution

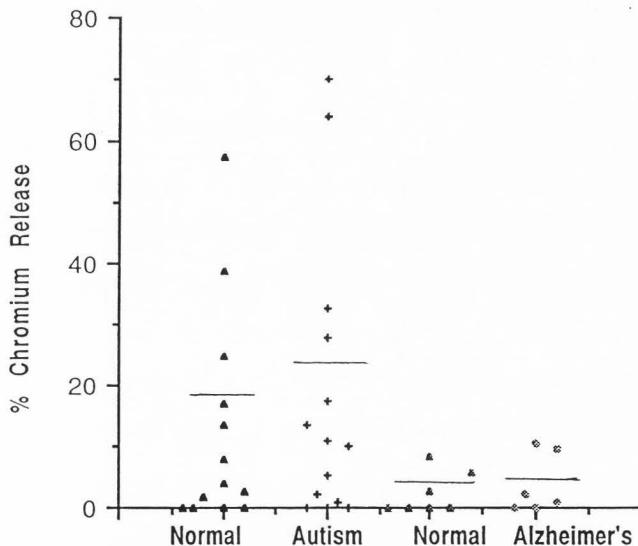


Figure 17 Untreated plasmas diluted 1:256.

Individual cytotoxic activities, expressed as percentages of chromium release, are shown for 4 different groups. The mean value for the controls age-matched to the autistic group (▲) was 18.6% (N = 13). For the autistic patients (+) it was 23.2% (N = 14). For the controls age-matched to the Alzheimer's group (▲) it was 5.5% (N = 7). For the Alzheimer's patients (\*) it was 5.7% (N = 6).

Cytotoxicity of Untreated  
Plasma at a 1/512 Dilution

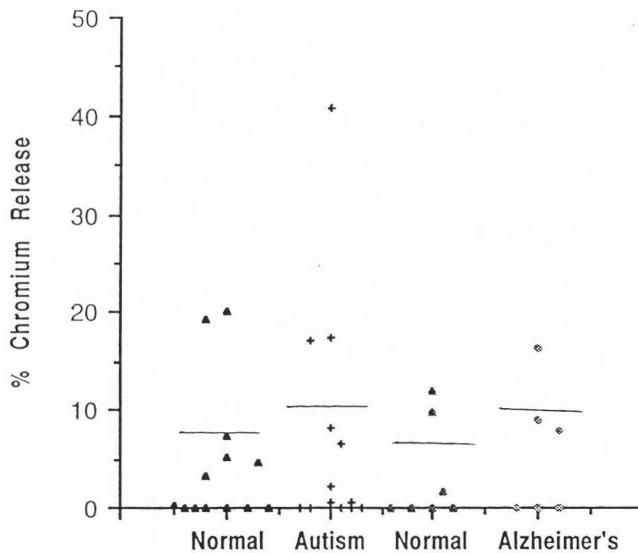


Figure 18 Untreated plasmas diluted 1:512.

Individual cytotoxic activities, expressed as percentages of chromium release, are shown for 4 different groups. The mean value for the controls age-matched to the autistic group (▲) was 8.6% (N = 13). For the autistic patients (✖) it was 11.6% (N = 14). For the controls age-matched to the Alzheimer's group (\*) it was 7.8% (N = 7). For the Alzheimer's patients (○) it was 11.0% (N = 6).

### Cytotoxicity of Heat-Treated Plasma at a 1/64 Dilution

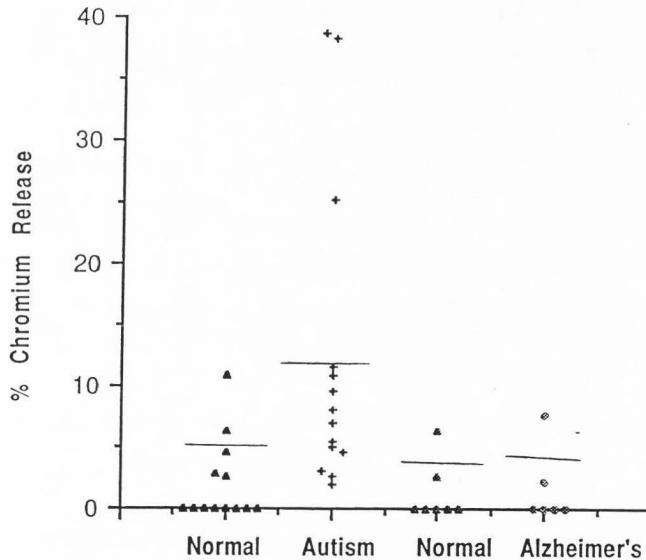


Figure 19 Heat-treated plasmas diluted 1:64.

Individual cytotoxic activities, expressed as percentages of chromium release, are shown for 4 different groups. The mean value for the controls age-matched to the autistic group ( $\blacktriangle$ ) was 5.5% (N = 13). For the autistic patients (+) it was 12.3% (N = 14). For the controls age-matched to the Alzheimer's group ( $\triangle$ ) it was 4.5% (N = 7). For the Alzheimer's patients (●) it was 4.9% (N = 6).

Cytotoxicity of Heat-Treated  
Plasma at a 1/128 Dilution

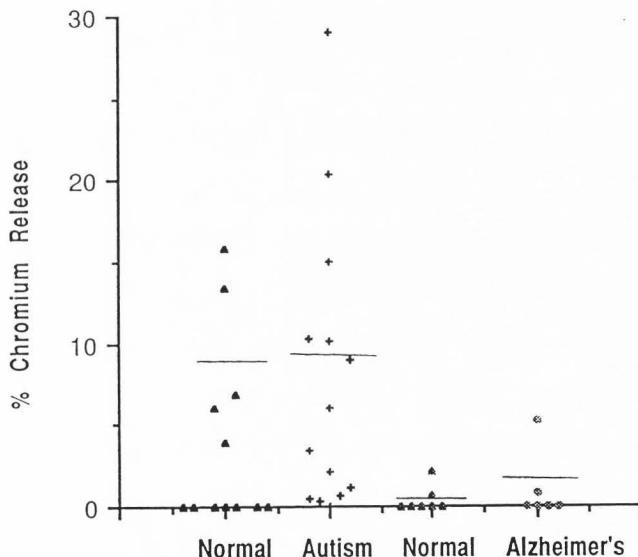


Figure 20 Heat-treated plasmas diluted 1:128.

Individual cytotoxic activities, expressed as percentages of chromium release, are shown for 4 different groups. The mean value for the controls age-matched to the autistic group (▲) was 9.2% (N = 13). For the autistic patients (+) it was 8.5% (N = 14). For the controls age-matched to the Alzheimer's group (\*) it was 1.4% (N = 7). For the Alzheimer's patients (\*) it was 2.6% (N = 6).

Cytotoxicity of Heat-Treated  
Plasma at a 1/256 Dilution

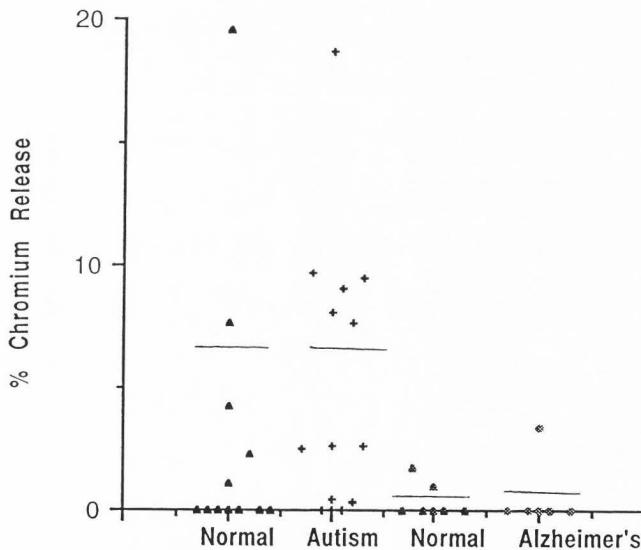


Figure 21 Heat-treated plasmas diluted 1:256.

Individual cytotoxic activities, expressed as percentages of chromium release, are shown for 4 different groups. The mean value for the controls age-matched to the autistic group (▲) was 7.0% (N = 13). For the autistic patients (+) it was 6.5% (N = 14). For the controls age-matched to the Alzheimer's group (▲) it was 1.4% (N = 7). For the Alzheimer's patients (\*) it was 1.7% (N = 6).

Cytotoxicity of Heat-Treated  
Plasma at a 1/512 Dilution

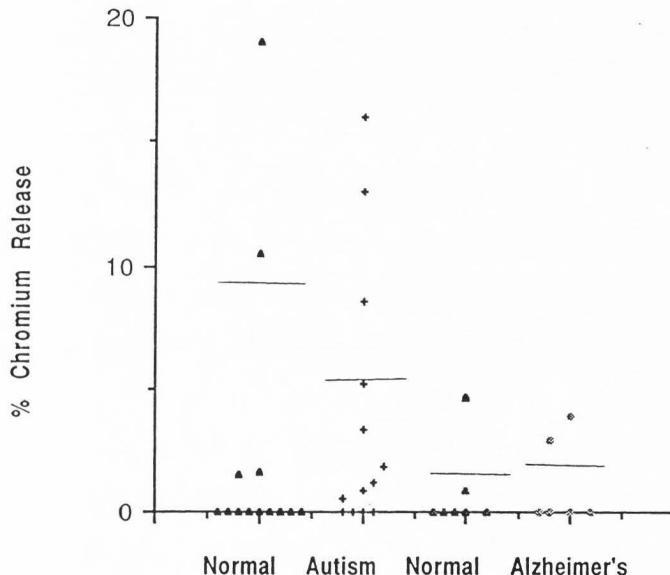


Figure 22 Heat-treated plasmas diluted 1:512.

Individual cytotoxic activities, expressed as percentages of chromium release, are shown for 4 different groups. The mean value for the controls age-matched to the autistic group (▲) was 8.2% (N = 13). For the autistic patients (✖) it was 5.6% (N = 14). For the controls age-matched to the Alzheimer's group (▲) it was 2.8% (N = 7). For the Alzheimer's patients (✖) it was 3.4% (N = 6).

Cytotoxicity of Rabbit Complement  
Treated Plasma at a 1/64 Dilution

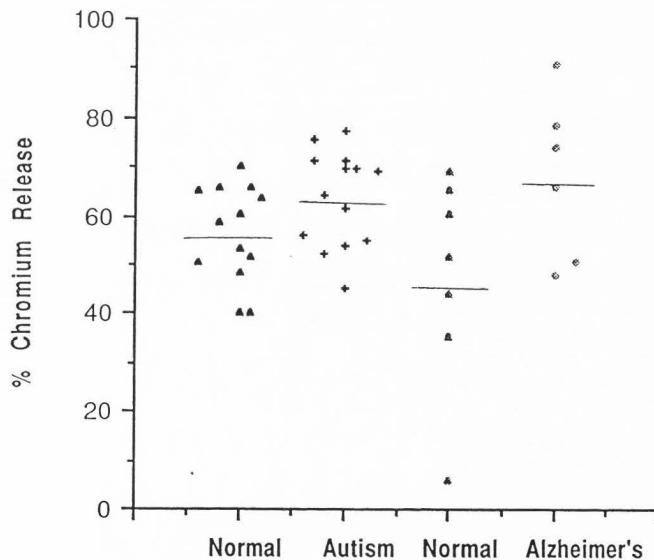


Figure 23 Complement-treated plasmas diluted 1:64.

Individual cytotoxic activities, expressed as percentages of chromium release, are shown for 4 different groups. The mean value for the controls age-matched to the autistic group (▲) was 56.4% (N = 13). For the autistic patients (+) it was 63.9% (N = 14). For the controls age-matched to the Alzheimer's group (\*) it was 47.2% (N = 7). For the Alzheimer's patients (\*) it was 67.8% (N = 6).

Cytotoxicity of Rabbit Complement  
Treated Plasma at a 1/128 Dilution

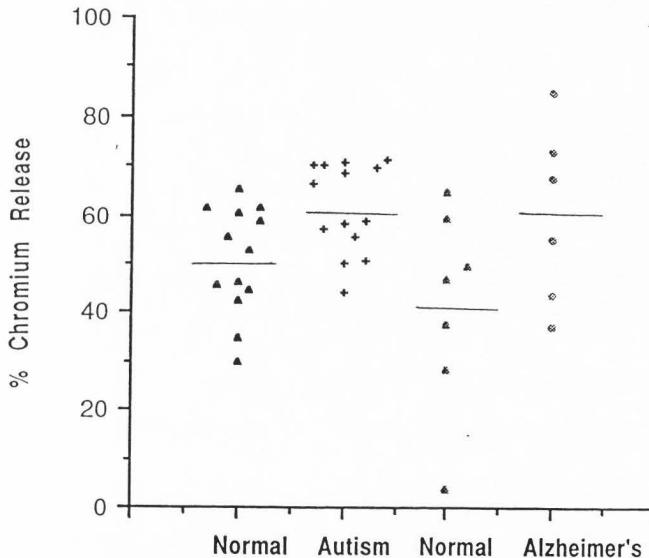


Figure 24 Complement-treated plasmas diluted 1:128.

Individual cytotoxic activities, expressed as percentages of chromium release, are shown for 4 different groups. The mean value for the controls age-matched to the autistic group ( $\blacktriangle$ ) was 50.7% ( $N = 13$ ). For the autistic patients (+) it was 60.3% ( $N = 14$ ). For the controls age-matched to the Alzheimer's group ( $\blacktriangle$ ) it was 41.4% ( $N = 7$ ). For the Alzheimer's patients (\*) it was 60.1% ( $N = 6$ ).

Cytotoxicity of Rabbit Complement  
Treated Plasma at a 1/256 Dilution

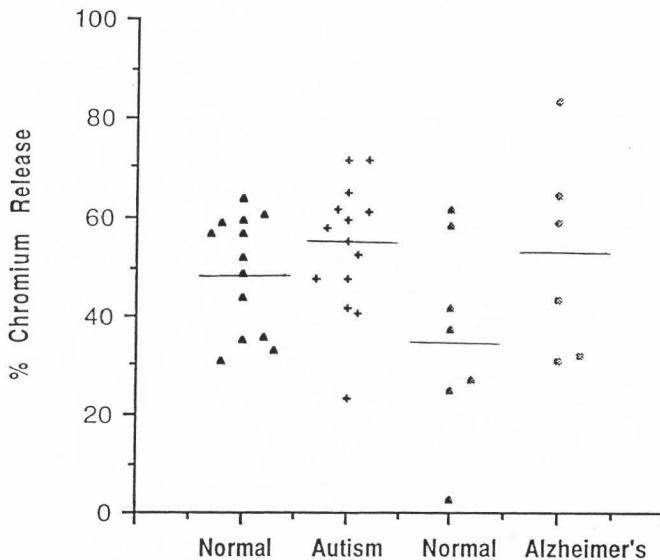


Figure 25 Complement-treated plasmas diluted 1:256.

Individual cytotoxic activities, expressed as percentages of chromium release, are shown for 4 different groups. The mean value for the controls age-matched to the autistic group ( $\blacktriangle$ ) was 48.8% ( $N = 13$ ). For the autistic patients (+) it was 55.2% ( $N = 14$ ). For the controls age-matched to the Alzheimer's group ( $\blacktriangle$ ) it was 36.3% ( $N = 7$ ). For the Alzheimer's patients (\*) it was 52.1% ( $N = 6$ ).

Cytotoxicity of Rabbit Complement  
Treated Plasma at a 1/512 Dilution

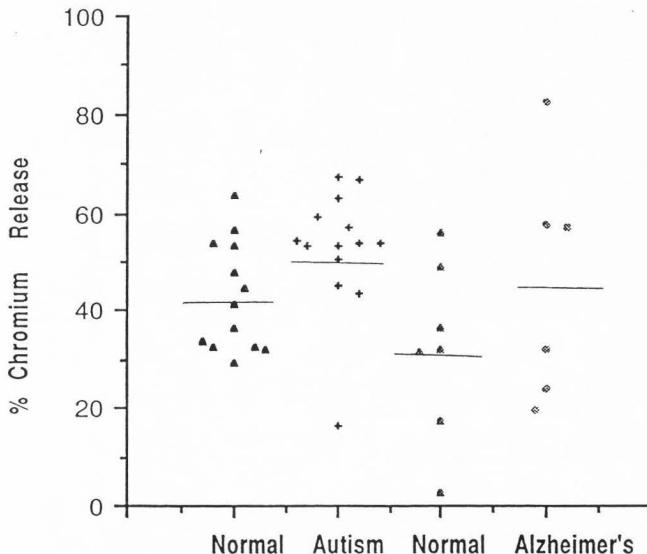


Figure 26 Complement-treated plasmas diluted 1:512.

Individual cytotoxic activities, expressed as percentages of chromium release, are shown for 4 different groups. The mean value for the controls age-matched to the autistic group ( $\blacktriangle$ ) was 42.9% ( $N = 13$ ). For the autistic patients (+) it was 52.0% ( $N = 14$ ). For the controls age-matched to the Alzheimer's group ( $\blacktriangle$ ) it was 32.1% ( $N = 7$ ). For the Alzheimer's patients (◎) it was 45.5% ( $N = 6$ ).

## DISCUSSION

A number of immune abnormalities occur in autistic and Alzheimer's patients. Several, but not all, of these defects appear to be related to the functions of T lymphocytes. The thymus, as an organ which controls the differentiation and maturation of T lymphocytes, could be a target for pathological and clinical manifestations seen in autism and AD. In this regard, plasmas from autistic patients and Alzheimer's patients were studied for reactivity with thymic cells. Though human thymic cell lines were ordered from the American Type Tissue Culture Collection (Rockville, MD), three successive attempts to grow them were unsuccessful. Because of their availability, murine thymic cells were used instead of human thymic cells. Three different methods were designed to study the possible existence of circulating antibodies to thymic tissue antigens.

Because of increased sensitivity of the ELISA method as compared with the indirect immunofluorescent method, the former method was used in this study. Significantly higher absorbance values were detected in the ELISA using plasmas of autistic subjects as compared to those of matched controls. These data indicate the possible existence of antithymic antibodies in the subgroups of autistic patients. More autistic samples would be needed to confirm the observation.

Findings with antibodies to thymic tissues relate with previous findings in autism such as lower CD4+ and CD45RA+

(Warren et al., 1990; Yonk et al., 1990), and lower response to T cell mitogens (Stubbs et al., 1977). Antithymic antibodies could be causing these abnormalities. Antibodies to neuron-axon filament proteins and myelin basic protein could also be causing these abnormalities (Singh & Fudenberg, 1988b).

The positive result ( $P < 0.001$ ) was observed in Alzheimer's patients compared to the normal controls. Previously, Singh and Fudenberg (1986) showed the existence of circulating antibodies to brain tissue antigens in AD. These brain antibodies also cross-reacted with thymic cells. The positive binding between murine thymocytes and plasma antibodies of Alzheimer's patients, and the observation from Singh and Fudenberg (1986) suggest that the immunoreactive antigen might be a common antigen in the brain and thymus.

The circulating antibodies in plasma of autistic patients did not inhibit the blastogenesis of thymic cells when compared to the matched controls. Also, no significance was observed when comparing the Alzheimer's patients to the matched group.

Because antithymic antibodies were observed with the ELISA, inhibition of thymic-cell proliferation was expected. Lack of inhibition may indicate that the *in vitro* binding of the antithymic antibodies observed was not strong enough to inhibit the cultured thymic cells. One reason the thymic cells were not inhibited might be that the antibody titer was

too diluted in the assay. More concentrated antibody may be needed, especially if the affinity of the human antibody binding to murine thymocytes was very weak. Another reason may be that the surface antigen of the thymic cells reaction in the ELISA was not important for *in vitro* thymic cell proliferation. For example, binding of the antibodies to the target surface antigens on the thymic cells was not a sufficient hinderance to the function of the cells, not allowing the antibodies to "neutralize" cell growth.

In plasma of autistic patients, no significant complement-dependent cytotoxic activity against thymic cells was observed when compared to the normal plasma under untreated and heat-treated conditions. However, significant cytotoxic activity of plasmas was observed when rabbit complement was added to the treated plasma samples.

These data indicate that the cytotoxicity of circulating antibodies to murine thymocytes observed in autistic patients was complement-dependent. No significant cytotoxicity of circulating antibodies to murine thymocytes was found in Alzheimer's patients under different conditions. These data pointed to the existence of circulating antibodies using the ELISA method, but no cytotoxicity to thymocytes.

Because antithymic antibodies were observed with the ELISA, higher levels of cytotoxicity were expected in plasma of autistic subjects. However, significantly higher levels of cytotoxicity ( $P = 0.043$  and  $0.013$  respectively) in patient

plasmas were seen only at dilutions of 1/64 and 1/128 in autistic patient plasmas and when rabbit complement was added. The failure of more dilute plasma of autistic subjects to cause cytotoxicity may be because these autistic subjects have increased frequencies of the C4B null allele (Warren et al., 1991).

One factor in control plasmas that may have caused high levels of cytotoxic activity is a human plasma killer protein, such as human complement. This protein may have been toxic to the murine thymic cells. In the heat-treated plasma experiments the toxic factor was heat labile. When rabbit complement was added to the heat-treated human plasmas, the high levels of cytotoxicity were restored. Therefore, the high levels of cytotoxicity in the plasmas of controls were probably caused by human complement, acting against murine thymic cells.

Future research using human thymocytes should study these abnormalities. It is not known what percentage mouse antithymic antibodies cross-react with human thymus tissue.

## CONCLUSIONS

The significant dose-related binding between murine thymocytes and plasma antibodies of autistic patients indicates the existence of antithymic antibodies which might react with surface antigens of human thymocytes, forming immune complexes. The detection of antithymic antibodies in plasmas from patients with AD, and the finding (Singh & Fudenburg, 1986) of a cross reaction of anti-brain antibodies to thymic antigens, suggests that there might be a common epitope in the brain and thymus of patients with Alzheimer's disease (AD).

These circulating antibodies in either autistic or AD patients did not significantly inhibit DNA synthesis of murine thymocytes when compared to normal age-matched controls. In addition, no significant cytotoxic activities of antithymic antibodies were detected using untreated plasmas or heat-treated plasmas from either autistic or AD patients.

However, when rabbit complement was added to heat-inactivated plasmas of autistic patients, significant cytotoxic activities of the antithymic antibodies were observed. These results implied that complement-dependent cytotoxicity, at least in autistic patients, was involved in the cytotoxic activity of the circulating antibodies harmful to murine thymocytes. Therefore, an immune dysfunction that allows aberrant production of autoantibodies may play a role

in the immune abnormalities of T lymphocyte reactions in autism.

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