IMMUNOPHENOTYPING OF LYMPHOID CELLS IN AUTISM

by

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I would like to dedicate this thesis to my parents, Richard and Nancy Yonk, and my grandfather, Merlin Yonk, for giving me a desire to learn about this world in which I live. Through their actions and example, they have shown me that I can do anything I want with my life. They have helped me believe in myself with their love, support and encouragement. I want them to know that I appreciate the sacrifices they have made for me over the years so that I could attain my goals.

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ABSTRACT

Immunophenotyping of Lymphoid Cells in Autism

by

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Research into the cause of autism continues without any clear-cut answers. However, recent studies suggest that abnormalities of the immune system are associated with this disorder and autism results from failure of the immune system to regulate itself. Proper immune regulation requires that the host have the appropriate number and percentage of each population of lymphocytes and monocytes. These populations can be distinguished from one another with monoclonal antibodies that react with unique protein structures on the cell surface designated as the "cluster of differentiation" (CD) antigens.

This investigation studied the possibility that the immune abnormalities seen in autism are due to a change in the lymphocytes or monocytes in the subjects with autism. The autistic subjects as compared to age- and sex-matched control subjects exhibited several changes in their cell populations. These included a depression of total lymphocytes, CD2+ (total T cells), CD4+ (helper T cells), and CD4+CD45RA+ (the antigen-inexperienced, suppressor inducer subset of helper T cells).

Also analyzed were the siblings of the autistic subjects. A reduced percentage of CD4+ cells was seen in the male siblings as compared to unrelated males. Analyses also compared the cells of mothers and fathers of the autistic subjects with controls of mothers and fathers of normal children. No differences were seen in any of the markers used.

Findings in the literature show an increase in memory cells and a decrease in naive cells as a function of age. The data gathered in these experiments uphold this concept and are consistent with the idea that CD45RA and CDw29 are maturational states of helper T
The quantitation of different immune markers on lymphoid cells seems to have been useful in the further characterization and investigation of the immune mechanism relevant to the syndrome of autism. Differences in some of the cell types were observed and may account for some of the immune abnormalities seen in autism. These differences may be the result or the cause of the syndrome. Further investigation seems necessary before a direct pathological link can be found between the body's immunity and autism.
CHAPTER I
INTRODUCTION

Autism is a neurobehavioral disorder that occurs throughout the world and shows no apparent predilection for race, culture, or social background. Autism has an onset before the age of 36 months and occurs in approximately four to five of every 10,000 births (DeMyer et al., 1973; Ritvo and Freeman, 1977; Steffenburg et al., 1989; Wing, Yeates, Brierley, and Gould, 1976). The syndrome is about four times more common in boys than in girls, and the subjects afflicted with autism face a lifelong disorder with a poor chance of ever leading an independent life.

The National Society for Autistic Children formulated a definition of the syndrome of autism. Its essential features included disturbance of developmental rates and sequences; unacceptable responses to sensory stimuli; problems with speech, language-awareness and nonverbal communication; and inability to relate appropriately to people, events, and objects (Ritvo and Freeman, 1977). The American Psychiatric Association (APA) used these criteria for the definition of autism as outlined in the Diagnostic and Statistical Manual of Mental Disorders (DSM) (American Psychiatric Association, 1980; American Psychiatric Association, 1987).

The cause of autism is unknown; however, current research suggests several causative factors including genetic, virologic, and immunologic. Twin studies have shown that genetics may play a role in autism (Folstein and Rutter, 1977). Ritvo et al., (1989), after studying multiple incidences in families, concluded there may be a genetically influenced subtype of autism. Problems with pregnancies and infertility have been observed in mothers of autistic subjects. Moreover, congenital rubella virus and/or congenital cytomegalovirus (CMV) infections have been associated with autism (Chess, 1977; Markowitz, 1983; Stubbs, 1978; Stubbs, 1987; Stubbs, Ash, and Williams, 1984).

Abnormalities of several immune parameters have been seen in patients with autism. Some autistic subjects have an abnormal response to mitogens in the blastogenesis assay (Singh, Fudenberg, Emerson, and Coleman, 1988; Stubbs, Crawford, Burger, and Vandenbark, 1977; Warren, Foster, Margaretten, Pace, and Thain, 1985; Warren, Foster,
Margaretten, and Pace, 1986), an inversion of the ratio of helper to suppressor T cells (Warren et al., 1985, 1986), a decrease in the total number of T lymphocytes (Warren et al., 1985, 1986; Warren, Yonk, Burger, Cole et al., 1990), and a reduction in the activity of natural killer cells (Warren et al., 1985; Warren, Foster, and Margaretten, 1987). Inhibition of macrophage migration occurred in some autistic patients' lymphocytes when they were incubated with human myelin basic protein, a component of myelin sheath (Weizman, Weizman, Szekely, Wijsenbeek, and Livni, 1982). Circulating antibodies against putative serotonin brain receptors have been found in some autistic patients (Todd and Ciaranello, 1985). The values for Om+ T cells, an immunoregulatory protein, were abnormally distributed in the autistic patients when compared with sibling controls (Singh et al., 1988). Another finding showed an irregular distribution in many autistic patients' autologous mixed-lymphocyte reaction (AMLR) when compared to siblings (Singh et al., 1988). Moreover, there is evidence for circulating antibodies against neural tissue antigens, for example, neuron-axon filament proteins, in many subjects with autism (Singh et al., 1988).

The two lobes of the cerebellum have been shown to be abnormally small, as detected by the technique of magnetic resonance imaging (MRI), in autistic children (Courchesne, Yeung-Courchesne, Press, Hesselink, and Jernigan, 1988). Another finding that has been reported is a significant decrease in the numbers of Purkinje cells in the cerebellar hemisphere and vermis of autistic subjects (Ritvo et al., 1986).
CHAPTER II
LITERATURE REVIEW

Introduction to Autism

Dr. Leo Kanner, professor of child psychiatry at Johns Hopkins School of Medicine, first introduced the term autism to the child psychiatric literature in 1943. In his paper "Autistic Disturbances of Affective Contact," he described a group of patients who did not form normal relationships, were upset by any change in their environment, and showed abnormalities in speech and language (Kanner, 1943). The adjective autistic was introduced into the adult psychiatric literature by Eugen Bleuler in describing self-referential qualities of certain thought processes (Blueler, 1950). References to the term autism as a disease or specific illness began in the literature between the 1940s and 1950s. Many labels have been associated with this syndrome and related disorders over the years. These include infantile autism, childhood schizophrenia, childhood psychosis, symbiotic psychosis, atypical ego development, severe emotional disability, early (primary) or late (secondary) onset autism, and mental retardation with autistic features. Much of the earlier confusion in the literature resulted from this general lack of classification or diagnostic guidelines.

Definition of Autism

The Professional Advisory Board of the National Society for Autistic Children (NSAC), in 1978, drafted a "Definition of the Syndrome of Autism" to help clarify the classification of autism (Ritvo and Freeman, 1977). A different but similar and overlapping definition of autism was formulated in 1980 for the third edition of the American Psychiatric Association's "Diagnostic and Statistical Manual" (DSM-III), revised in 1987, and is now regularly updated as new research data continue to accumulate (American Psychiatric Association, 1980; American Psychiatric Association, 1987).

Autism is a behaviorally defined syndrome because objective pathognomic biomedical markers common to all cases have not yet been determined. It is a rare happening since it occurs in only about four or five of every 10,000 births (DeMyer et al., 1973; Ritvo and
Autistic males outnumber autistic females four fold. Autism happens in all parts of the world with no apparent prevalence for any race, culture, or social background (Ando and Tsuda, 1975; Kolvin, 1971; Spence, Simmons, Brown, and Wikler, 1973).

An essential feature of autism is that it is manifested before 36 months of age. There are two reported courses for the development of the illness. In the first, abnormal behavior is noted within the first few months of life, and these infants are either very good babies who never fuss or very irritable babies who overrespond to any stimuli. Children who follow the second pathway have relatively normal development until around 18 to 24 months of age, at which time the parents first begin to notice symptoms (Freeman and Ritvo, 1984; Ornitz and Ritvo, 1968; Rutter, 1967).

Autism is a lifelong disorder in which the symptoms may fluctuate, abate, and even disappear, but the initial diagnosis is maintained. Those few autistic subjects who live a normal life span need periodic reevaluations since their symptoms may disappear or reappear with age. The prognosis for autistic children, however, is poor, and 60-75% of autistic subjects lead a life of complete or semi-dependence (DeMyer et al., 1973; Rutter, Greenfield, and Lockyer, 1967).

Prognosis for Autistic Subjects

From 7% to 28% of autistic subjects who showed no evidence of neurological disorders develop seizures by age 18 (Creak, 1963; Rutter, 1970). These subjects tend to be severely retarded, underdeveloped, and impaired. Failure to develop communication skills or the ability to play with or use toys by the age of 5 is associated with a very poor prognosis (Brown, 1960). Findings show a better prognosis for autistic children who develop motor skills and communicative language before the age of 5. However, during adolescence the subjects in this group become very shy, introverted, and passive and may show signs of a disturbed reality typical of schizophrenia (Bender, 1969; Bender, 1970). Very few autistic children lead a life of semi-independence and get employment. Those who do still have problems relating with others and are handicapped in their social and cognitive skills
as children and adults (Brown, 1963; Gaijzago and Prior, 1974).

**Symptoms of Autism**

The rates and/or sequences of development in autistic subjects are disturbed, and the normal coordination of the three developmental pathways (motor, social-adaptive, cognitive) are disrupted. One or more of the pathways may have delays, arrests, or regressions within it. Characteristic are sequences of spurts and plateaus in development (Bender and Freedman, 1952; Fish, 1960).

Autistic children show disruption in their responses to sensory stimuli and an alteration of generalized hyporeactivity and hyperreactivity ranging from hours to months (Goldfarb, 1961; Goldfarb, 1963). Disturbances involving visual stimuli include close scrutiny of visual details, avoidance of eye contact, staring, prolonged observation of hands or objects, and attention to changing levels of illumination (Bender, 1947; Bender, 1956). The close regard to self-induced sounds and nonresponse or overresponse to varying sound levels are characteristic of problems with auditory stimuli (Anthony, 1958; Condon, 1975). Tactile symptoms include over or underresponsiveness to touch, pain, and temperatures, extended rubbing of surfaces, and sensitivity to the texture of food. A preoccupation with spinning objects is illustrative of vestibular disturbances, including over- or underreactions to gravity stimuli and twirling without dizziness. Problems seen in olfactory and gustatory stimuli include sniffing repetitively, specific food preferences, and licking objects that are not edible. Posturing, darting-lunging movements, hand-flapping, gesticulations, and grimaces are all part of the disturbances seen in the proprioceptive stimuli (Ritvo and Freeman, 1978; Sorosky, Ornitz, and Brown, 1968).

Impairment in speech, language, and cognitive capacities occur in autistic subjects. Speech may be totally absent (mutism) or have a delayed onset with immature syntax and articulation (Bender, 1947; Kanner, 1943; Wolff and Chess, 1965). Once functional speech develops it is often atonal and arrhythmic, lacking inflection or emotion (Goldfarb, 1961). Autistic subjects show absent or limited symbolic capacity in language-knowledge. They do not develop the use of abstract terms, concepts, and reasoning. Echolia is a common feature
with or without communicative intent. Frequently there is a misuse or reversal of pronouns with the echolia (Kanner, 1943; Wing, 1966). Concepts are nonlogically used by the autistic subjects. They often misuse words or invent new words that have no meaning. The nonverbal communication skills of autistic subjects are also lacking, with an absent or delayed development of appropriate gestures, dissociation of gestures from language, and failure to determine symbolic meanings from gestures.

There are disturbances in the capacity of children with autism to relate to people, events, and objects. They show poor eye contact; develop a delayed social smile; have a slow response to being picked up; react with aversion to physical contact; respond to only a part of another person; show no interest in playing games with others; and, to strangers, show a delayed, absent, or overreactive anxiety response (Hutt and Ounsted, 1966; Kanner, 1943; Ornitz and Ritvo, 1976; Wing, 1966). Cooperative play and friendships, which usually appear between the ages of 2 and 4, may not appear. Shallow cursory responses to adults and peers in response to strong social cues may develop in autistic children between the ages of 5 and 7. Autistic children frequently do not use toys or objects in an age-appropriate manner and fail to assign them symbolic or thematic meanings. They habitually flick, twirl, or spin toys instead of using them in their proper way (Eisenberg, 1956; Kanner, 1943). Autistic children are upset at the disruption of any kind of routine that they have established. They seem to be aware of the sequence of events, and any disruption of this sequence makes them panic. They want to maintain "sameness" in their environment. As autistic children grow older, they become worse at relating to people, places, and events (Frith, 1971). Disturbances of thought, mood, and behavior vary with age in autistic subjects. There may be unexplained crying; laughing for no apparent reason; lack of appreciation of danger; inappropriate fears; hair pulling, biting, or other self-damaging behaviors; and repetitive stereotypic movement of limbs or the entire body (Freeman and Ritvo, 1984).

There is extreme variability of intellectual functioning on formal IQ tests given to children with autism. Estimates are that about 60% of autistic children have an IQ score of below 50; 20% score between 50 and 70; and 20% score at 70 or above. A score of 70
or below on IQ tests indicates mental retardation (Goldberg and Soper, 1963; Rutter, 1970). Autistic children do best on tests of manipulative or visual-spatial skills and rote memory. They perform very poorly on anything calling for abstract thought, symbolism, or sequential logic. Most autistic children show wide variations in performance on different tests at different times (Ritvo and Freeman, 1978).

Proposed Theories Explaining Autism

Autism may follow many different pathways in development of the syndrome. Genetic, virologic, and/or immunologic bases are some suggestions. Studies in twins have shown that monozygotic pairs are likely to be concordant while dizygotic pairs are discordant for autism (Folstein and Rutter, 1977). The analysis of multiple incidences in families has led to the proposal that a subtype of autism may be genetically influenced (Ritvo et al., 1989).

Virologic and immunologic contributions to autism seem closely tied together. Susceptibility/resistance to viral infections and other immunological relationships, including autoimmunity, are exercised by the major histocompatibility complex (Stubbs, Ritvo, and Mason-Brothers, 1985). Parents of autistic children have a significant increase in their incidence of infertility and have at least two more spontaneous abortions compared to the general population (Funderburk, Carter, Tanguay, Freeman, and Westlake, 1983). The histocompatibility complex has been linked to other abnormalities of pregnancy including recurrent abnormal pregnancies of unknown cause (Gerencer, Kastelan, Drazancic, Kerhin-Brkljacicic, and Madjaric, 1978), neural tube defects (Schacter, Gyves, Muir, and Tasin, 1979), toxemia of pregnancy (Redman, 1980), and midtrimester bleeding (Peterson and Torrey, 1976). The problems with bleeding could be interpreted as secondary to an immunological attack. Many studies have shown that the firstborn is the likeliest to be affected (Deykin and MacMahon, 1980; Pittfield and Oppenheim, 1964; Tsai and Stewart, 1983; Wing, 1966). It is not uncommon for subjects with autism to be smaller than average in size (Campbell et al., 1980; DeMyer, Ward, and Lintzenich, 1968; Dutton, 1964; Simon and Gillies, 1964). Viewing autism as a mild form of graft-versus-host disease could account
for this smaller size (Palm, 1974).

In pregnancy, it may be better for the father and mother to share very few HLA markers as these differences stimulate the blocking antibodies that protect the fetoplacental unit (Bee, Quebbeman, Ayers, and Haines, 1981). This happens although the mother's lymphocytts still recognize and are sensitized to paternal alloantigens. The blocking factors work by either preventing recognition or inhibiting the effector arm of the immune response. The blocking factors have been isolated (Rocklin, Kitzmiller, Carpenter, Garovoy, and David, 1976), but the precise mechanism by which they work is unknown. Many women with recurrent abortions appear to lack blocking antibodies in their sera, though they do produce cell-mediated responses to paternal alloantigens. Theoretically, then, the fetus is unprotected and is aborted. In other cases, the mother may be reacting against specific antigens of spermatozoa, resulting in cytotoxic effects. The brain and spermatozoa share antigens (Erickson, 1977). The central nervous system has several cell surface antigens that react with spermatozoa and no other tissues. Instead of the placental tissues being affected, leading to the abortion, the central nervous system of the fetus might be affected, perhaps leading to autism (Stubbs et al., 1985).

A higher incidence of autism occurs in children with congenital rubella (Chess, 1977) and/or congenital cytomegalovirus (CMV) infections (Markowitz, 1983; Stubbs, 1978; Stubbs, 1987; Stubbs et al., 1984). In addition, many subjects with autism fail to produce rubella antibodies, despite rubella vaccine immunization (Stubbs, 1976). Congenital CMV could be the culprit in many more cases of autism than is reported, because most pregnant mothers never know that they are infected, and one out of every 100 live-born babies shed CMV virus in their urine, though most are asymptomatic (Stubbs, 1987). Problems such as learning disorders, mental retardation, deafness, hyperactivity, and sociobehavioral disorders can occur in a significant number of children born with asymptomatic CMV (Stubbs, 1987).

Immune abnormalities found in autistic people include abnormal response in the T-lymphocyte blastogenesis assay to phytohemagglutinin (PHA), concanavalin A (Con A), and pokeweed mitogen (PWM); alteration in the ratio of helper to suppressor T cells; a decrease in the number of T lymphocytes as assessed by the rosette assay; and a depression in the
activity of natural killer cells (Singh et al., 1988; Stubbs et al., 1977; Warren et al., 1985, 1986, 1987; Warren, Yonk, Burger, Cole et al., 1990). Protection against malignancy and viral infections, and regulation of the immune response is mediated by both T cells and natural killer cells. Abnormalities in both T cells and natural killer cells are also found in autoimmune disorders (Abo and Balch, 1983; Alpert, Turek, Foung, and Engleman, 1987; Edwards et al., 1987; Minota and Winfield, 1987; Morimoto et al., 1987; Morimoto, Abe, and Homma, 1979; Reinherz, Rubinstein, Geha et al., 1979; Reinherz and Schlossman, 1980; Strelkauskas, Callery, McDowell, Borel, and Schlossman, 1978). Autistic subjects compared to their siblings have shown an irregular response to Om+ T cells and an abnormal response in the autologous mixed lymphocyte reaction (AMLR). The latter may result from failure of their suppressor T cells to function normally (Singh et al., 1988). In addition, there are reports that some autistic patients make a cell-mediated immune response that is inappropriate to myelin basic protein, a component of myelin sheath (Weizman et al., 1982). However, the leukocyte-migration inhibition assay used in this study did not isolate any autoantibodies against myelin basic protein. An autoimmune response against myelin basic protein has been suspected to play a pathological role in multiple sclerosis (Waksman, 1981). A similar autoimmune response may be important in the pathogenesis of autism, and a preliminary finding of autoantibodies to myelin basic protein may be relevant to this hypothesis (Singh et al., 1988).

Magnetic resonance imaging (MRI) studies have found defects in the cerebellum of autistic subjects. The average areas for cerebellar vermal lobes VI and VII in autistic subjects was 249.4 square millimeters, while the control subjects averaged 304.9 square millimeters (Courchesne et al., 1988). The total number of Purkinje cells in the cerebellar hemisphere and vermis were also significantly reduced in autistic subjects (Ritvo et al., 1986).

There is evidence that autoimmunity may play a role in the syndrome of autism. A very casual association between autism and autoimmune disease has been reported (Money, Bobrow, and Clarke, 1971). They thought that the central nervous system might be affected by autoantibodies. In contrast to control groups, circulating antibodies to
serotonin receptors in the brain were present in 30% of autistic children tested (Todd and Ciaranello, 1985). Antibodies to neuron-axon filament protein are also present in autistic subjects (Singh et al., 1988). An increase in alcoholism, psychiatric illness, and mental retardation has been identified in the families of autistic patients (Lobascher, Kingerlee, and Gubbay, 1970). Antinuclear antibodies plus thyroid were increased in autistic children over those in controls (Stubbs, 1987). Stubbs also found a significantly higher frequency of CMV antibodies in autistic girls when compared to autistic boys. Mother-child pairs that tested higher are significantly higher than normal mother-child pairs (Stubbs, 1987).

Flow Cytometric Analysis of Immune Cells

With the introduction of a highly sophisticated technology, known as flow cytometry, it has been possible to propose the characterization and the isolation of cells, especially of white blood cells (immune cells), with a much greater sensitivity, accuracy, simplicity, and rapidity than conventional fluorescence microscopy techniques. Moreover, many difficulties inherent in the fluorescence microscopy, such as subjectivity and inability to quantitate fluorochrome intensity on different cells, are overcome when using flow cytometry.

A cell preparation analyzed by flow cytometry uses four parameters: size, granularity, and red and green fluorescence (Coulter Immunology, 1986). As individual viable cells, stained by conventional immunofluorescence techniques, pass through a laser beam, analysis occurs for forward angle light scatter (FLS), 90 degree light scatter (90LS), and fluorescence at two different wavelengths distinct from the light source; usually green (GFL) and red (RFL) fluorescence. The laser beam illuminates the sample particle as it flows within a laminar sheath. This classification of cells into categories is more objective, because the criteria for denoting a specific subpopulation comes from the histograms and is not done visually by the researcher.

When light hits a particle (whether a cell, cell nuclei, or other microscopic particle) in a fluid, light is scattered from the particle in all directions (angles) from the incident beam (Melamed, Mullaney, and Mendelsohn, 1979; Pinkel and Stovel, 1985). The amount of light scattered is a function of the size and shape of the particle. The light scattered in
a forward direction for spheres with physical characteristics similar to cells is related to both the volume and refractive indices (Hodkinson and Greenleaves, 1963; Mullaney, 1970). The size of the particle is related to the light scattered at narrow angles, while the light scattered at 90 degrees relates to the granularity or internal structure of the cell (Benson, McDougal, and Coffey, 1984; Brunsting and Mullaney, 1974; Mullaney and Dean, 1970; Visser, van den Engh, and van Bekkum, 1980). Flow cytometry uses these two light scatter measurements to define and isolate subsets of a particle population (Coulter Immunology, 1986; Lovett et al., 1984; Salzman et al., 1975). Light scattering can also separate out contamination of the sample by other cell types. Less light is scattered by red blood cells, which causes their peak to be to the left of the main lymphocyte population. Granulocytes, monocytes, and polymorphonuclear leukocytes in the cell population can also be separated and analyzed because they scatter more light and so will be in a peak to the right of the smaller leukocytes (Sucic et al., 1989). Scatter alone can differentiate nonviable lymphocytes and other types of cells from the rest of the cell population, thereby excluding them from the analysis and separation of the samples (Loken and Herzenberg, 1975).

Light is absorbed at one wavelength in the molecules of a fluorescent dye, and then the high energy light (shorter wavelength) converts into chemical energy and lower energy light (longer wavelength). Each fluorescent dye has a characteristic excitation and emission spectra; that is, when a narrow band of wavelengths of light is absorbed, it produces a narrow band of emitted light (fluorescence) (Martin, Rolland, Nairn, and Muller, 1982). Flow cytometry exposes a fluorescently labeled cell population to a wavelength of light within the labeling dye’s excitation spectrum and determines the presence and relative amount of dye on each particle. The amount of light reaching a detector is configured by filters to accept only the wavelengths of light in the dye’s emission spectra. In this way the system can detect and isolate subsets of a sample population based on the presence of fluorescently labeled cell components (Loken, 1980; Loken, Parks, and Herzenberg, 1977; Loken and Stall, 1982).

Requirements for the analysis of an individual particle in a sample population are that the particles be separated from each other so that they are distinguishable, and that
the light source be small enough in cross section so that the illumination of two particles simultaneously is prevented (Coulter Immunology, 1986). A flowing sheath draws the particles out one from another; thus, they are separated from each other. These separated cells flow in single file through the center of the cross section that is illuminated by a very narrowly focused laser beam. The point at which the laser and particle meet should, ideally, be the intersection of three Gaussian distributions: laser intensity, particle position, and sensor sensitivity (Shapiro, 1988). After the particle flows through the cross section and is illuminated by the laser, the light scatter and fluorescence signals are detected and converted to analog voltage pulses which are amplified and converted to digital values. This accumulation and storage on a particle-by-particle basis obtains the distribution of the measured characteristics of the cell population (Matsui, Shapiro, Sheehy et al., 1986; Matsui, Staunton, Shapiro, and Yunis, 1986).

A No-Wash Method of Processing Cells

A standard practice for immunofluorescent staining for either fluorescence microscopy or flow cytometry has been to repeatedly wash the samples to remove any unbound fluorescent markers. This was done to remove the background fluorescence that might interfere with the determination of positively stained cells. It now appears that repeated washings may interfere with certain markers in diseased patients and produce erroneous results (Caldwell and Taylor, 1986). No-wash techniques are now being developed for immunophenotypic analysis using flow cytometry. One system is the Coulter Immunoprep Epics Leukocyte Preparation System. It is a gentle, rapid erythrocyte lysing system that needs no washing or centrifugation, and maintains leukocyte morphology and cell surface markers with good recovery. It has three components: an erythrocyte lysing agent, a leukocyte stabilizer, and a cell membrane fixative. Refrigeration will keep samples for analysis up to 24 hours after processing with the Immunoprep system (Coulter Immunology, 1986).
Overview of T Lymphocytes

T lymphocytes, despite their morphological similarities, display an extraordinary degree of heterogeneity of function including proliferation to cell surface antigens and soluble antigens, cytotoxic killing activity in cell-mediated lympholysis, and helper and suppressor functions. It appears that precursor bone marrow cells (prothymocytes) migrate to the thymus gland, where they are processed. After they are functionally competent they are exported into the peripheral lymphoid compartments, which includes the spleen, lymph nodes, and blood (Moore and Owen, 1967; Owen and Raff, 1970; Owen and Ritter, 1969; Stutman and Good, 1971). The various stages of T cell ontogeny are marked by profound changes in cell surface antigens (Konda, Stockert, and Smith, 1973; Raff, 1971). A series of now developed monoclonal antibodies for thymocytes and/or peripheral T lymphocyte cell surface antigens help in the identification of some different subpopulations existing in T lymphocytes. Monoclonal antibody markers developed for cell surface antigens on B lymphocytes further identify the subclasses of the B lymphocyte population.

Monoclonal Antibodies to Human Lymphocytes

In Table I on page 14 is a summary of the monoclonal antibodies described in this thesis. Most of the monoclonal antibodies have been described using "CD" designation numbers that are universally used.

The human T helper cells, which represent 33 to 54% of normal human T lymphocytes, are defined by a monoclonal antibody called CD4 (T4). Approximately 80% of thymocytes have the CD4 antigen marker. The molecular weight of the CD4 associated antigen is 64,000 daltons. The CD4 antigen is stable and is not lost upon activation (Terhorst, van Agthoven, Reinherz, and Schlossman, 1980). The CD4+ cells are the only cells that (1) respond to soluble antigens, (2) help to develop the optimal cytotoxicity in cell-mediated lympholysis, and (3) are required in a pokeweed mitogen (PWM) driven system to cause B cell proliferation and immunoglobulin production (Reinherz, Kung, Breard, Goldstein, and Schlossman, 1980; Reinherz, Kung, Goldstein, and Schlossman, 1979a).
# Table I. Summary of Monoclonal Antibodies to Various Lymphocytes and Monocytes

<table>
<thead>
<tr>
<th>Name</th>
<th>Target Cell Population</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. T4+ (CD4)</td>
<td>Helper T lymphocytes</td>
</tr>
<tr>
<td>2. T8+ (CD8)</td>
<td>Suppressor/cytotoxic T lymphocytes</td>
</tr>
<tr>
<td>3. T11+ (CD2)</td>
<td>Total T lymphocytes</td>
</tr>
<tr>
<td>4. B1+ (CD20)</td>
<td>Total B lymphocytes</td>
</tr>
<tr>
<td>5. NKH-1+ (CD56)</td>
<td>Cells with natural killer activity</td>
</tr>
<tr>
<td>6. T4+4B4+ (CDw29)</td>
<td>Helper-inducer subset of T helper lymphocytes; Memory maturational state of helper T cell</td>
</tr>
<tr>
<td>7. T4+2H4+ (CD45RA)</td>
<td>Suppressor-inducer subset of T helper lymphocytes; Naive maturational state of helper T cell</td>
</tr>
<tr>
<td>8. MO2 (CD11b)</td>
<td>Adherent monocytes and macrophages</td>
</tr>
<tr>
<td>9. KC56 (T-200) (CD45)</td>
<td>Leukocyte common antigen</td>
</tr>
<tr>
<td>10. IL-2R1 (CD25)</td>
<td>Interleukin 2 receptor</td>
</tr>
<tr>
<td>11. I3+</td>
<td>Monocytes, macrophages, B lymphocytes, and activated T lymphocytes</td>
</tr>
<tr>
<td>12. Mouse IgG</td>
<td>Control for cross-reactivity between human cells and monoclonal antibodies</td>
</tr>
</tbody>
</table>
Immunoregulatory abnormalities can be identified using the CD4 marker. It is useful in
detecting and studying juvenile rheumatoid arthritis, graft-versus-host disease, systemic
lupus erythematosus, and acquired immunodeficiency syndrome (AIDS) (Alpert et al., 1987;
Dalgleish et al., 1984; Fauci et al., 1985; Morimoto, Reinherz, Schlossman et al., 1980;
Reinherz, Cooper, and Schlossman, 1981; Reinherz, Geha, Wohl et al., 1981; Smolen et al.,
1982).

A subset of human cells with cytotoxic and suppressor activities has been defined
by a monoclonal antibody called CD8. The CD8 (T8) antigen is found on 80% of thymocytes
and 17-48% of peripheral T lymphocytes in normal humans. The antigen CD8 has a
molecular weight of 33,000 daltons in the reduced state and 76,000 daltons under non-
reduced conditions (Terhorst et al., 1980). Anti-CD8 is unreactive with normal B cells, null
cells, and macrophages. Proliferation was seen when cells that had this marker were
incubated with the mitogen concanavalin A (con A) or other alloantigens. After sensitization
in MLC, the CD8+ subset contained cytotoxic effector cells, and after stimulation with con
A, contained suppressor effector cells. Cells with the CD8 marker did not react to soluble
antigen (Reinherz et al., 1979a; Reinherz, Kung, Goldstein, and Schlossman, 1980; Reinherz
and Schlossman, 1980). Many autoimmune diseases including multiple sclerosis, juvenile
rheumatoid arthritis, and systemic lupus erythematosus have been associated with a
decrease in cells positive for the CD8 marker (Alpert et al., 1987; Morimoto, Reinherz, Abe,
Homma, and Schlossman, 1980; Morimoto, Reinherz, Schlossman et al., 1980; Reinherz,
Geha, Wohl et al., 1981; Reinherz, Weiner, Hauser et al., 1980; Smolen et al., 1982;
Streikauskas et al., 1978). An increase in CD8+ cells is identifiable with disease states in
infectious mononucleosis, cytomegaloviral disease, herpes zoster, and measles (Reinherz,
Cooper, and Schlossman, 1981). Problems with the mechanisms of turning off activation of
the CD8 lymphocytes have been seen in chronic graft-versus-host disease and lepromatous
leprosy (Mehra et al., 1980; Reinherz, Parkman, Rappeport, Rosen, and Schlossman, 1979).

CD2 (T11) is an antigen associated with the erythrocyte (E) rosette receptor, occurs
as a single polypeptide chain with a molecular weight of 50,000 daltons and is found on all
thymocytes, peripheral T cell, and some null cells, but not on B cells (Howard et al., 1981).
Clinically, the CD2 marker can aid in the quantitation of the total T lymphocytes in circulating blood (both immature and mature) (Kamoun et al., 1981). Decreased total T lymphocytes are associated with autoimmune diseases such as systemic lupus erythematosus, multiple sclerosis, and severe eczema (Morimoto et al., 1982; Reinherz and Schlossman, 1981). Increased numbers of T cells are identified with patients with infectious mononucleosis (Reinherz and Schlossman, 1981).

The CD20 (B1) antigen has a molecular weight of 35,000 daltons and is found on all B cells isolated from the peripheral blood, lymph nodes, spleen, tonsils, and bone marrow. The CD20 antigen is not found on monocytes, resting or activated T cells, null cells, or tumors of T cell and myeloid origin. The elimination of all CD20 positive cells in the peripheral blood removed the population of cells induced to differentiate into Ig-secreting plasma cells in a pokeweed mitogen (PWM) driven system (Stashenko, Nadler, Hardy, and Schlossman, 1980). Patients with systemic lupus erythematosus have an increased number of CD20 positive cells (Morimoto et al., 1982).

An antigen with a molecular weight of 200,000 is called CD56 (NKH-1) and is expressed on large granular lymphocytes (LGL) which includes cells with natural killer activity. It is not found on B cells, T cells, erythrocytes, granulocytes, monocytes, or platelets (Herberman and Ortaldo, 1981). CD56 is expressed on approximately 4 to 26% of the peripheral blood mononuclear cells (Hercend et al., 1985). The proportion of CD56+ cells increases with age, and cord blood from neonates contains 1% or less of CD56+ cells. The proportion of CD56+ cells then increases throughout childhood and into adult life (Abo and Balch, 1983). Higher levels of CD56+ cells are found in men (Abo and Balch, 1982). Natural killer cells may mediate natural resistance to such things as tumors, viral infections, and bone marrow allografts (Hercend et al., 1986; Reinherz, O'Brien, Rosenthal, and Schlossman, 1980). In the absence of antibodies or prior immunization, natural killer cells are able to lyse certain tumor cells in vitro (Griffin, Hercend, Beveridge, and Schlossman, 1983).

The lymphocytes positive for CD4 have two distinct subsets. One of these subsets is the helper-inducer subset characterized by the CD4+CDw29+ antibodies. The CDw29 (4B4) surface antigen has a molecular weight of 135,000 daltons. It is present on 41% of
unfractionated peripheral T lymphocytes, 41% of peripheral CD4 lymphocytes, 43% of peripheral CD8 lymphocytes, 5-30% of monocytes, and over 30% of null cells and monocytes. Poor proliferation is seen when CD4+CDw29+ cells are stimulated by concanavalin A or alloantigens. However, stimulation by soluble antigens results in good proliferation and because of this the CD4+CDw29+ subset of cells provides a good helper signal for the detection of PWM-induced Ig synthesis (Morimoto, Letvin, Distaso, Aldrich, and Schlossman, 1985; Morimoto, Letvin, Boyd et al., 1985; Reinherz et al., 1982; Reinherz, Geha, Wohl et al., 1981; Reinherz, Kung, Breard et al., 1980; Reinherz, Kung, Goldstein, and Schlossman, 1979b; Smolen et al., 1982). This subset is also believed to represent the memory maturational state of the helper T cell (DePaoli, Battistin, and Santini, 1988; Pirruccello, Collins, Wilson, and McManus, 1989; Sanders, Makgoba, and Shaw, 1988; Tedder, Cooper, and Clement, 1985; Warren, Yonk, Burger, and Singh, 1990). The joints of rheumatoid arthritics have recently been found to have CD4+CDw29+ cells present, perhaps accounting for the inflammatory process (Alpert et al., 1987; Morimoto, Reinherz, Schlossman et al., 1980). The CD4+CDw29+ marker is also important in the study of the prognosis for AIDS, autoimmune, and other immune deficiency patients (Reinherz, Cooper, and Schlossman, 1981; Reinherz, Geha, Wohl et al., 1981; Smolen et al., 1982).

The lymphocyte surface antigen CD45RA (2H4) with a molecular weight of 190,000 daltons characterizes the suppressor inducer subset of CD4 positive lymphocytes. This marker is shown on 42% of unfractionated peripheral T lymphocytes, 41% of peripheral CD4 lymphocytes, 54% of CD8 lymphocytes, 5-30% of monocytes, and over 30% of peripheral B cells and null cells. Con A stimulation causes the CD4+CD45RA+ subset to proliferate well. Soluble antigen, however, is given poorly by this subset as is help to B cells. Most importantly, the CD4+CD45RA+ subset induces CD8 cells (Morimoto, Letvin, Distaso et al., 1985; Reinherz et al., 1979b; Reinherz et al., 1982; Reinherz, Kung, Breard et al., 1980; Reinherz and Schlossman, 1980). It is now believed that this subset also represents the naive maturational state of the helper T cell (DePaoli et al., 1988; Pirruccello et al., 1989; Sanders et al., 1988; Tedder et al., 1985; Warren, Yonk, Burger, and Singh, 1990). Clinically, a decrease in CD4+CD45RA+ cells has been associated with systemic lupus
erythematous, juvenile rheumatoid arthritis, and multiple sclerosis (Alpert et al., 1987; Morimoto et al., 1987; Morimoto et al., 1981; Reinherz, Cooper, and Schlossman, 1981; Reinherz, Geha, Wohl et al., 1981; Smolen et al., 1982).

Adherent monocytes and macrophages derived from peripheral blood have an antigen called CD11b (MO2) present on more than 70% of their cells (Todd, Nadler, and Schlossman, 1981). A medium-to-large mononuclear cell, the monocyte is found in bone marrow and peripheral blood and upon appropriate stimulation will differentiate into larger free macrophages. These macrophages are commonly found at sites of inflammation and in peritoneal, pleural, and synovial fluids. Monocytes/macrophages are important in the immune response because they process antigenic material and display it on their cell surface in a form that provides a signal to T lymphocytes, initiating antigen-specific pathways of immune function. A direct cytotoxic role against microorganisms, other mononuclear cells, and tumor cells may also be one of the functions of macrophages. They also may help natural killer cells destroy human tumor cells (Todd and Schlossman, 1982). In contrast to these positive effects, the macrophage may also play another role in immune regulation and suppress some immunological functions, possibly by elaborating inhibitory factors (Allison, 1978).

All cells except mature erythrocytes and their immediate progenitors express CD45 (T-200) (KC56), the leukocyte common antigen (Coffman and Weissman, 1981; Dalchau and Fabre, 1980; Dalchau, Kirkley, and Fabre, 1981; Omary, Trowbridge, and Battifora, 1980). Because it is found on thymocytes, bone marrow cells, peripheral blood lymphocytes, macrophages, and granulocytes, CD45 is useful in determining the accuracy of gated human leukocyte populations on 90 degree versus forward angle light scatter. When used in conjunction with CD11b the evaluation and quantitation of distinct leukocyte populations for three-part differential analysis is possible. It shows the populations of lymphocytes, monocytes, and granulocytes (Newman, Targan, and Fast, 1984; Todd et al., 1981).

The receptor for interleukin 2, CD25 (IL-2R1), is defined by a glycoprotein with a molecular weight of 60,000 daltons. Mitogen or antigen activated T and B cells exhibit the CD25 receptor. The receptor is not expressed on resting T cells thymocytes, or null cells.
(Fox et al., 1984). Cells with increased numbers of CD25 receptors occur in some acute viral infections (Fox et al., 1985).

Human monocytes, macrophages, B lymphocytes, and activated T lymphocytes express the antigen I3. It is a nonpolymorphic class II Ia antigen of undetermined molecular weight. Greater than 75% of peripheral blood monocytes express I3. Ia-negative cells, including resting T lymphocytes, granulocytes, erythrocytes, and platelets are all negative for the I3 antigen. Class II Ia antigen-restricted immune interactions, like antigen-presentation, allogeneic and autologous mixed leukocyte responses, generation of T cell cytotoxicity, and cytotoxicity produced by CD4+CDw29+ subsets are inhibited by anti-I3 antibody. The antigen marker I3 can aid in diagnosis of viral infections by identifying the states of T lymphocyte activation (Todd, Meuer, Romain, and Schlossman, 1984).
CHAPTER III
MATERIALS AND METHODS

Subjects

Blood was drawn during early morning hours from 25 autistic children ranging in age from 3 to 31 years with a mean age of 11.0 years. The autistic children included in the study consisted of 18 males, ranging in age from 3 to 24, with a mean age of 9.5 years; and 7 females, ranging in age from 6 to 31, with a mean age of 14.8 years. The immediate family members of the autistic subjects were also included in this study. They consisted of 25 siblings (12 males with a mean age of 10.3 years, and 13 females with a mean age of 8.8 years) ranging in age from 2 to 18, with a mean age of 9.5 years; 20 mothers ranging in age from 25 to 49, with a mean age of 37.2 years; and 20 fathers ranging in age from 29 to 56, with a mean age of 41.2 years. The control families consisted of 20 age-matched subjects (11 males with a mean age of 8.4 years, and 9 females with a mean age of 14.8 years), ranging in age from 2 to 25 years, with a mean age of 11.3 years; 12 mothers ranging in age from 24 to 48, with a mean age of 36.0 years; and 12 fathers ranging in age from 24 to 49, with a mean age of 35.0. The autistic children included did not have Down's syndrome, and met DSM-IIIIR criteria as evaluated by at least one private physician and confirmed by a clinical psychologist. To satisfy DSM-IIIIR guidelines, the children had to comply with at least eight of sixteen criteria included in three different groups. Two of the criteria had to be met in the first group on reciprocal social interaction. These criteria were: a marked lack of awareness of feelings of others, no or abnormal seeking of comfort at times of distress, no or impaired imitation behavior, no or abnormal social play, and gross impairment in ability to make peer friendships. In the second group on verbal and nonverbal communication and imaginative play, at least one of the criteria had to be met. The criteria were: no mode of verbal communication, markedly abnormal nonverbal communication, absence of imaginative activity, marked abnormalities in speech production, marked abnormalities in form or content of speech, and marked impairment in the ability to initiate or sustain a conversation, despite adequate speech. At least one criteria in the
third group, which included a repertoire of activities and interests, had to be met. This group included: stereotyped body movements, persistent preoccupation with parts of an object, marked distress over changes in trivial aspects of the environment, unreasonable insistence on following routines in precise detail, and a markedly restricted range of interests. Only children who were diagnosed with early infantile autism before 36 months of age were included in the study. All subjects had idiopathic autism, and only one was receiving medications (tegretol for control of seizures); none had concurrent medical problems that might affect the results of this study. All autistic subjects had been under the care of a physician since the original diagnosis of their disease, and none had any sign of infection and/or allergies when their blood was drawn for this study. All subjects were living at home at the time of the study.

**White Blood Cell Counts**

Whole blood, 0.01 ml, was pipetted in vials containing 10 ml of Isoton 2 (Coulter Immunology, Hialeah, Fl). To this sample a drop of lysing and hemoglobin reagent was added. The cells were counted on a Coulter counter (Coulter Electronics, Hialeah, Fl) and the white blood cell count recorded. After percentages were obtained for cells expressing each specific marker using flow analysis, these values were used to determine the absolute numbers of lymphocytes expressing each marker.

**Preparation of Cells for Flow Cytometric Analysis**

Whole blood, 0.1 ml, was pipetted into a 12 x 75 mm test tube and to this 0.01 of the appropriate antibody preparation was added. The antibodies used include anti-T4 (CD4), helper T cell; anti-T8 (CD8), suppressor and cytotoxic T cells; anti-T11 (CD2), total T cells; anti-B1 (CD20), all B cells; anti-NKH-1 (CD56), natural killer cells; anti-T4+4B4+ (CDw29), memory, helper inducer T cells; anti-T4+2H4+ (CD45RA), naive, suppressor inducer T cells; anti-IL-2R1 (CD25), activated T cells; anti-I3, monocytes, macrophages, B cells and activated T cells; anti-MO2 (CD11b), adherent monocytes and macrophages; anti-KC56 (CD45), leukocyte common antigen, all cells except erythrocytes; and as a control for any
background, non-specific staining mouse anti-IgG (Coulter Immunology). The whole blood and antibodies were incubated for ten minutes at room temperature after which they were fixed using the Immunoprep instrument (Coulter Immunology) on the 35 second cycle. The samples were analyzed immediately or stored in the refrigerator for up to 24 hours before flow analysis.

**Flow Analysis**

Dual-color fluorochrome analysis was done on the Epics-C flow cytometer (Coulter Electronics) equipped with an argon laser for 488 nm wavelength. Eleven monoclonal antibodies and the negative control of biotinylated nonimmune mouse IgG were analyzed for each person. Cells were carried in Isoflow (Coulter Electronics) sheath fluid through a 76 micron flow tip. Fluorescence data for fluorescein-isothiocyanate (FITC) and phycoerythrin (PE) were obtained, using a bit-map format gated on the forward light scatter versus right angle histogram. Fluorescence intensity was standardized using latex beads of 10um (Coulter Electronics) and adjusting the laser power to place the log-green histogram in channel 119. For each sample 2000-5000 cells were analyzed on a logarithmic scale. The percentage of lymphocytes as a function of the total white blood cell count was determined by differential gating on the Log green versus Log red fluorescence histogram after dual-color staining with the monoclonal antibodies Mo2-PE (Coulter Immunology) and KC56 (T-200)-FITC (Coulter Immunology). The percentages for each of the ten monoclonal antibodies was saved for further analysis.

**Related Experiments**

Other research was done while this investigation was ongoing. The changes in naive and memory T cell populations were found using flow cytometry. Data from all investigations were combined to make the findings statistically significant.

**Statistical Analyses**

All data were analyzed using the analysis of covariance and the student's t test.
CHAPTER IV
RESULTS

Comparisons of Autistic Subjects with Healthy Controls

The results of analysis of peripheral blood mononuclear cells of autistic subjects and healthy controls are summarized in Tables II and III. Compared to the healthy control group, the group of 25 autistic subjects showed a significantly reduced (p < 0.05) percentage and absolute number of total lymphocytes, as well as decreased (p < 0.05) number of CD2+ cells, a depressed (p < 0.05) number and percentage of CD4+ cells, and a decreased (p < 0.05) number of CD4+CD45RA+ cells. The number and percentage of CD8+, CD20+, CD4+CDw29+, CD56+, CD25+, I3+ lymphocytes, I3+ monocytes and total number of monocytes were unchanged in the autistic subjects as compared to the healthy subjects.

Further Breakdown of Autistic Group into Males Versus Females

Separate analysis on the number and percentages of various cells in male and female autistic subjects revealed that both sexes had reduced numbers and percentages of all the cell populations and subpopulations that were decreased in the combined group. The females, however, seemed to have a much greater reduction overall than the males as shown in Table II. Also, when compared with the healthy controls, only the females in the group were significantly different (p < 0.05) from the autistic subjects. The males in the healthy control group showed no significant differences in any of the markers except in the percentages and absolute numbers of CD4+ (p < 0.05).

Comparisons with the Autistic Group and Their Siblings

The comparison of the autistic group with their siblings showed differences in some of the markers including decreases (p < 0.05) in the percentages and absolute numbers of the total number of lymphocytes, the percentage and total number (p < 0.05) of CD4+, the total number (p < 0.05) of CD2+, and the total number (p < 0.05) of CD4+CD45RA+ as seen in Tables II and III. However, when separate analysis was done with each sex it was found
**Table II. Summary of Immunophenotyping Data**

<table>
<thead>
<tr>
<th>Lymphocyte type</th>
<th>Autistic Subjects (n = 25)</th>
<th>Siblings (n = 25)</th>
<th>Healthy Controls (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male (9.5) *</td>
<td>Female (14.8)</td>
<td>Total (11.0)</td>
</tr>
<tr>
<td>Total lymph</td>
<td>2777 (209)</td>
<td>2246 (247)</td>
<td>2628 (215)</td>
</tr>
<tr>
<td></td>
<td>49 *</td>
<td>39 *</td>
<td>46 *</td>
</tr>
<tr>
<td>CD2+</td>
<td>2124 (161)</td>
<td>1703 (204)</td>
<td>2006 (173)</td>
</tr>
<tr>
<td></td>
<td>76 *</td>
<td>76 *</td>
<td>76 *</td>
</tr>
<tr>
<td>CD4+</td>
<td>1100 (114)</td>
<td>832 (123)</td>
<td>1025 (115)</td>
</tr>
<tr>
<td></td>
<td>40 *</td>
<td>37 *</td>
<td>39 *</td>
</tr>
<tr>
<td>CD8+</td>
<td>655 (60)</td>
<td>540 (67)</td>
<td>623 (62)</td>
</tr>
<tr>
<td></td>
<td>24 *</td>
<td>24 *</td>
<td>24 *</td>
</tr>
<tr>
<td>CD20+</td>
<td>462 (41)</td>
<td>304 (53)</td>
<td>418 (45)</td>
</tr>
<tr>
<td></td>
<td>17 *</td>
<td>14 *</td>
<td>16 *</td>
</tr>
<tr>
<td>CD4+</td>
<td>569 (157)</td>
<td>385 (113)</td>
<td>716 (23)</td>
</tr>
<tr>
<td>CD45R+</td>
<td>27 *</td>
<td>17 *</td>
<td>23 *</td>
</tr>
<tr>
<td></td>
<td>385 (113)</td>
<td>716 *</td>
<td>911 *</td>
</tr>
<tr>
<td>CD4+</td>
<td>279 (68)</td>
<td>229 (66)</td>
<td>341 (66)</td>
</tr>
<tr>
<td>CDw29+</td>
<td>14</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>(68)</td>
<td>(66)</td>
<td>(66)</td>
</tr>
</tbody>
</table>

a. Mean age of subjects in the group; standard error of the mean is given in the parentheses.
b. Mean and standard error of the mean of the total number of cells/mm³.
c. Percentage of cells; lymphocytes as a function of total white blood cells and other populations as a function of total lymphocytes.
d. Total autistic group significantly lower (p < 0.05) than total sibling or healthy control groups.
e. Females in the autistic group are significantly lower (p < 0.05) than females in the sibling or healthy control groups.
f. Males in the autistic group are significantly lower (p < 0.05) than males in the healthy control group.
g. Males in the sibling group are significantly lower (p < 0.05) than males in the healthy control group.
### Table III. Further Summary of Immunophenotyping Data

<table>
<thead>
<tr>
<th>Lymphocyte type</th>
<th>Autistic Subjects (n = 25)</th>
<th>Siblings (n = 25)</th>
<th>Healthy Controls (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (11.0)a</td>
<td>Total (9.5)</td>
<td>Total (11.3)</td>
</tr>
<tr>
<td>CD25+</td>
<td>573b (103)</td>
<td>560 (122)</td>
<td>438 (83)</td>
</tr>
<tr>
<td></td>
<td>22c</td>
<td>17</td>
<td>14</td>
</tr>
<tr>
<td>I3+ Lymphocytes</td>
<td>591 (92)</td>
<td>603 (35)</td>
<td>635 (90)</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td>CD56+</td>
<td>152 (109)</td>
<td>209 (203)</td>
<td>194 (197)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Total Monocyte</td>
<td>208 (31)</td>
<td>178 (20)</td>
<td>1953 (19)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3</td>
<td>19</td>
</tr>
<tr>
<td>I3+ Monocyte</td>
<td>146 (23)</td>
<td>140 (15)</td>
<td>158 (16)</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>79</td>
<td>81</td>
</tr>
</tbody>
</table>

a. Mean age of subjects in the group.

b. Mean and standard error of the mean of the total number of cells/mm³.

c. Percentage of cells: lymphocytes as a function of total white blood cells and other populations as a function of total lymphocytes.
that the female siblings, and not the males siblings, were significantly different ($p < 0.05$) when compared to the autistic group.

**Comparisons of Autistic Siblings and Healthy Controls**

The comparisons of the siblings and the healthy controls showed no significant differences in any of the markers used, as can be seen in Tables II and III. However, when separate analysis according to sex was done, a significant difference ($p < 0.05$) was found in the percentage of CD4+ comparing the male siblings to the healthy males. When the absolute numbers of CD4+ were analyzed according to sex, the differences were not significant.

**Comparison of Parents in Each Group**

The mothers of the autistic children as compared to the healthy control mothers showed no differences upon comparison of percentage and absolute numbers of each of the markers. The fathers of the autistic children did not show any differences in percentages or total numbers of each marker when compared with the healthy control fathers.

**Changes in Helper T Subsets According to Age**

The CD4+CD45RA+ cells showed a gradual decline in number and percentage of cells with increasing age, while the CD4+CDw29+ cells showed a concomitant gradual increase in number and percentage as shown in Figure 1 and 2. These subsets are also known as naive (CD4+CD45RA+) and memory (CD4+CDw29+) cells. As mentioned earlier in this chapter the autistic subjects had a decreased number of CD4+CD45RA+ cells.
Fig. 1. Total number and percentage of total lymphocytes of CD45R+ and CDw29+ helper T (CD4+) cells.
Fig. 2. Mean and 1 standard deviation of CD45R+ and CDw29+ helper T cells in subjects of various age groups. Means of both subsets correlated with age; $r = 0.99$ for CD45R and $r = 0.89$ for CDw29.
CHAPTER V
DISCUSSION

The current study extends earlier findings that immune system abnormalities exist in patients with autism (Singh et al., 1988; Stubbs, 1976; Stubbs, 1987; Stubbs et al., 1977; Todd and Ciaranello, 1985; Warren et al., 1986; Weizman et al., 1982). A selective reduction or phenotypic change of CD4+ cells has been identified in this disease. Since it is well known that cells expressing the CD4 marker are involved in the regulation of antibody production and generation of suppressor T cell function (Reinherz and Schlossman, 1980; Reinherz et al., 1979a), it is possible that a lack of CD4+ cells in autistic subjects may account for some of the reported immune changes seen in this disease. For example, the failure of some autistic subjects to produce specific antibodies following rubella vaccination (Stubbs, 1976) and depressed lymphocytic responses to T cell mitogens (Singh et al., 1988; Stubbs et al., 1977; Warren et al., 1986) could be related to a decrease of helper T cells in autistic subjects. That only about 30% of the autistic subjects had reduced numbers of CD4+ cells is consistent with the concept that autism is a syndrome of subsets, and one subset thereof may involve depression of CD4+ cells.

The relevance of reduced CD4+ cell numbers in autism is not fully understood, but several possibilities exist. Viruses have been associated with autism (Chess, 1977; Stubbs, 1978; Stubbs, 1987). Therefore, it is possible that a deficiency of helper cells is associated with an increased rate of infections in autism. Such viral infections may involve the central nervous system. A second possibility is that the decreased number of circulating CD4+ cells in some autistic subjects could result from viral infection of the CD4+ cells. Supportive of this concept are findings that patients with acquired immunodeficiency syndrome (AIDS) have a depression in the number of circulating CD4+ cells (Fauci et al., 1985) and that the human immunodeficiency virus (HIV) selectively infects CD4+ cells and impairs their growth. The CD4 molecule on the surface membrane of CD4+ cells serves as a binding site for HIV. A similar decrease in CD4+ cells has been reported in other autoimmune disorders; for example, systemic lupus erythematosus (Morimoto et al., 1981), Sjogren’s syndrome.
(Morimoto et al., 1981), juvenile rheumatoid arthritis (Morimoto et al., 1981), and multiple sclerosis (Morimoto et al., 1987).

Another possibility is that there is a problem with the thymus where the T lymphocytes mature, and the cells are not able to differentiate properly. This could cause the subjects to never have had the correct number and percentages of the CD4+ and CD4+CD45RA+ cells.

The reduced percentage of CD4+ cells in the male siblings as compared to male control subjects was significant at the 95% confidence interval. This finding might be an evidence for a genetic relationship between CD4+ phenotypic expression in the autistic subjects and their siblings. However, the absolute number of CD4+ cells was not reduced in the male siblings, as it was in the male autistic subjects. The low number of male siblings is probably inadequate to validate these results.

The immunocompetent cells believed to be involved in the generation of suppressor cell function express the CD4+CD45RA+ marker (suppressor-inducer, naive helper T cells). It is, therefore, possible that a deficit of CD4+CD45RA+ cells in autistic subjects may also account for some of the described immune changes seen in this disease. For example, inappropriate cell-mediated immune responses to basic myelin protein (Weizman et al., 1982), and circulating antibodies directed against putative serotonin receptors (Todd and Ciaranello, 1985), could be associated with the lack of suppressor-inducer, naive T cells.

The CD4+CD45RA+ subset is also believed to represent the naive maturational state of T helper cells (DePaoli et al., 1988; Pirruccello et al., 1989; Sanders et al., 1988; Tedder et al., 1985; Warren, Yonk, Burger, and Singh, 1990). These cells are thought to decrease over age as exposure to more antigens occur. A concomitant increase in memory or CD4+CDw29+ cells is seen. The autistic subjects have a mean age of 11.0 years which is still fairly young to have such a decrease in naive cells. This would indicate that they have an impaired immune function in responding to previously unknown antigens. This could help explain why some autistic subjects failed to produce specific antibodies following vaccination with rubella (Stubbs, 1976) and some subjects show a depressed lymphocytic response to T cell mitogens (Singh et al., 1988; Stubbs et al., 1977; Warren et al., 1986).
It is unclear what relevance, if any, a decreased number of suppressor-inducer, naive T cells has on the development of autism, but several possibilities exist. A similar reduction of CD4+CD45RA+ cells has been reported in diseases widely accepted to be autoimmune disorders including systemic lupus erythematosus (Morimoto et al., 1987) and multiple sclerosis (Morimoto et al., 1987). The CD4+CD45RA+ cell deficiency may be associated with the development of cell-mediated or humoral responses against various elements of the central nervous system by allowing the activation of certain self-reactive T cell and B cell clones. Alternatively, there may be a modulation of expression of CD4 antigen on the cell-surface of T helper cells and sequestering of CD4+ cells in some extravascular site, for example, the central nervous system. The latter possibility may be tested by the detection of a lymphocytic infiltrate (containing mainly CD4+CD45RA+ cells) in the brain of autistic subjects. However, such examinations have thus far not been done, essentially because of the lack of availability of brain autopsies and/or biopsies from subjects with autism.

It is interesting that there was not a significant difference in CD56+ (cells with natural killer cell activity) cells in percentage or absolute number. One of the immune abnormalities observed was abnormal natural killer cell activity (Warren et al., 1985; 1987). The subjects number and percentage of these cells is normal, but the activity is not, and would then seem to indicate a problem with the immune regulation of these cells.

Analyses according to gender was done separately to see if any relationship existed between phenotypic expression and gender among the autistic subjects. There was some indication that the relative reductions and increase in the different cell markers was greater in females than in males. The analyses according to gender also found that the autistic group was only significantly different with the females of both the siblings and the healthy controls in all of the markers except CD4+. The basis for this gender difference in expression of the different markers is unknown, but, it could be related to the fact that autism occurs 3-4 times more frequently in males than in females.

The children in all the groups show some differences in percentages of some of the markers reported in the literature (Table IV) (Abo and Balch, 1983; Coulter Immunology, 1986; DePaoli et al., 1988; Fleisher, Hagengruber, and Marti, 1988; Lovett et al., 1984;
### Table IV. Proportionate Distribution of Various Cells in the Blood of Normal Subjects

<table>
<thead>
<tr>
<th>Cell Population or Subpopulation</th>
<th>Literature Values</th>
<th>Healthy Controls</th>
<th>Autistic Subjects</th>
<th>Autistic Siblings</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2+ (T11, Total T)</td>
<td>69-88</td>
<td>79</td>
<td>76</td>
<td>79</td>
</tr>
<tr>
<td>CD4+ (T4, Helper T)</td>
<td>33-54</td>
<td>47</td>
<td>39</td>
<td>42</td>
</tr>
<tr>
<td>CD8+ (T8, Suppressor/Cytotoxic T)</td>
<td>17-48</td>
<td>20</td>
<td>24</td>
<td>23</td>
</tr>
<tr>
<td>CD4+CD45RA+ (T42H4, Suppressor-Inducer, Naive)</td>
<td>9-23</td>
<td>29</td>
<td>23</td>
<td>27</td>
</tr>
<tr>
<td>CD4+CDw29+ (T44B4, Helper-Inducer, Memory)</td>
<td>14-34</td>
<td>13</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>CD20+ (B1, B lymphocytes)</td>
<td>11-18</td>
<td>16</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>CD56+ (NKH-1, Natural killer cell)</td>
<td>4-26</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>I3+ monocytes</td>
<td>&gt;75</td>
<td>81</td>
<td>70</td>
<td>75</td>
</tr>
</tbody>
</table>
Pirruccello et al., 1989; Sucic et al., 1989; Waxdal, Monical, Fleisher, and Marti, 1988). The helper subsets of CD4+CDw29+ (memory; helper-inducer) and CD4+CD45RA+ (naive; suppressor-inducer) are decreased and increased respectively over the values found in the literature. The mean ages of the autistic subjects was 11.0 years with a range of 3 to 31 years, the siblings mean age was 9.5 years with a range of 2 to 18 years, and the healthy controls had a mean age of 11.3 years with an age range of 2 to 25 years. It would seem then that a shift from mostly naive (suppressor-inducer) cells to mostly memory (helper-inducer) cells occurs as the children grow up. The adult populations studied had percentages well within the literature values. This observation has been published before (DePaoli et al., 1988; Pirruccello et al., 1989; Sanders et al., 1988; Tedder et al., 1985; Warren, Yonk, Burger, and Singh, 1990).

From Table IV it can be observed that I3+ monocytes should be expressed on at least 75% of the cells. The autistic subjects only express this monocyte population on 70% of their cells. This is not a significant result, however, when compared with the siblings of the autistic subjects or the healthy controls. It is interesting, though, because this is the part of the antigen receptor that recognizes self in presenting antigen to T lymphocytes (Todd et al., 1984). This would seem to indicate that the autistic subjects are not going to be able to present antigen as efficiently as they should and so will not start immune reactions very quickly. The lack of this marker for self recognition could be again due to a problem with differentiation, but this time in the bone marrow and with the monocytes. This could also affect the T cells, if the hemapoietic T progenitor cell was affected in the bone marrow, causing the failure to differentiate correctly in the thymus.

No significant differences were seen in total lymphocytes, total monocytes, CD2+, CD4+, CD8+, CD20+, CD56+, CDw29+, CD45RA+, CD11b+, CD45+, CD25+, and I3+ in either the fathers or the mothers of autistic subjects when compared to the healthy controls. The parents do not seem to have any immune abnormalities as far as phenotyping is concerned. There does not seem to be any familial inheritance of the decreases seen in autistic subjects.

Clearly, additional studies on the immune systems of autistic subjects need to be
done to substantiate these findings and explore the basis for the lymphocyte deficiencies seen in this severe developmental disorder. Family studies need to continue to confirm the results in this study, and to determine possible genetic transmission of these cellular deficiencies and increases.

It would be interesting to do functional immune studies to see if the decreases seen in the different cell types also effect the autistic subjects immune function. Genetic studies such as HLA typing could be done to see if there is genetic transmission from the parents of these cell deficiencies. Further studies on autoantibodies in the autistic child using flow cytometry, as well as gel electrophoresis, could also be interesting because of the decrease in the suppressor-inducer subset of the helper cells as seen in some autoimmune diseases.

In conclusion, it would seem that the decreases in the CD4+ (helper T cells) and CD4+CD45RA+ (naive; suppressor-inducer) lymphocytes in the autistic subjects would indicate a problem in immune regulation in these subjects. They have a decrease in a vital immune subset that helps to activate suppression. These problems in immune regulation could allow self-reactive T cell or B cell clones, or self reactive autoantibodies, to be made against the central nervous system or brain. This latter suggestion has been found to be the case in multiple sclerosis. They have autoantibodies reacting against their central nervous system and in particular their myelin sheath (Morimoto et al., 1987). The depressions in these subsets leading to problems with immune regulation could either cause the disease of autism or lead to its symptoms. Further investigations into the immune system in autistic subjects needs to be done to determine the significance of these findings and the role that they play in the disease.
REFERENCES


Bender, L. (1947). Childhood schizophrenia; Clinical study of 100 schizophrenic children. American Journal of Orthopsychiatry, 17, 40-56.


Fish, B. (1960). Involvement of the central nervous system in infants with schizophrenia. *Archives of Neurology,* 2, 115-121.


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behavior. *Archives of General Psychiatry, 18*, 439-449.


