ASSOCIATION OF THE MAJOR HISTOCOMPATIBILITY COMPLEX
WITH AUTISM

by

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

Association of the Major Histocompatibility Complex with Autism

by

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The pathogenesis of autism has proven difficult to characterize. However, in many recent studies, it is suggested that the onset of this disorder is the result of multiple etiological factors, which include genetic, immunologic, and viral elements.

Possible immunological influences found in subpopulations of patients with autism include decreased lymphocyte responsiveness, reduced natural killer cell activity, abnormal response to rubella vaccine, abnormal immune response to brain tissue, and decreased plasma levels of the fourth component of complement (C4). These aberrations and others imply a possible autoimmune mechanism in some cases for the development of autism.

C4 deficiencies have been found in subjects with established autoimmune disorders, such as systemic lupus erythematosus and chronic active hepatitis, in recent
investigations. There is also evidence that the major histocompatibility genes play an intimate role in autoimmune processes. Therefore, in knowing that the C4 genes are closely linked to the major histocompatibility genes, this study determined and analyzed the human leukocyte antigen profile of autistic patients, their siblings, and parents.

In this study, it was found that the C4B complement null allele occurred in autistic patients at nearly twice the frequency compared to normals. However, the C4A complement null allele frequency was not found to be significantly altered. Several extended haplotypes were represented within the patients studied. However, the extended haplotype B44-SC30-DR4 was the only one found at a significantly increased frequency. Further investigations are needed to better understand the significance of these findings.

(64 pages)
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Autism is a developmental disorder characterized by impaired social development and limited communication skills. A definitive etiological agent for this disorder has not yet been established; however, the pathogenesis of autism is likely a result of multiple factors based upon genetic, immunologic, and viral mechanisms. Some recent investigations suggest that autism demonstrates several features characteristic of established autoimmune disorders, such as genetic predisposition, presence of antibodies against brain antigens, sex differences (autism is four to five times more common in boys than in girls), and other immune abnormalities.

Many genes whose products are involved in various immune responses are found within the major histocompatibility complex (MHC) located on the short arm of the sixth human chromosome. The combination of genes found within the MHC is referred to as an MHC haplotype. Interestingly, it has been found that within the MHC, crossing over occurs at a reduced frequency, resulting in haplotypes remaining unchanged for many generations. A haplotype exhibiting this behavior has been termed an extended haplotype.

In Caucasians, extended haplotypes make up approximately 25-30% of all MHC haplotypes and are of importance because they are associated with various clinical manifestations.
For example, the extended haplotype HLAB*8, BF*S, C2*C, C4A*Q0, C4B*1, HLADR*3, abbreviated B8-SC01-DR3, is strongly associated with systemic lupus erythematosus (SLE). The actual pathogenic relationship between B8-SC01-DR3 and SLE remains obscure but a plausible explanation for this association may be the presence of a null allele at the C4A gene of this extended haplotype. A null allele is functionally silent (yields no functional gene product) and therefore results in reduced plasma levels of C4 protein. This C4 deficiency may hinder various complement functions including the capacity to prevent the precipitation, or proper clearing, of immune complexes.

Immune abnormalities have also been reported to be associated with the C4B locus. For instance, high anti-cytomegalovirus antibody titers (IgG isotype) have been observed in patients with reduced C4B plasma levels. Moreover, persons carrying two null alleles at the C4B loci have an increased number of infections of bacterial meningitis and with *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis* infections.

Low levels of complement C4 protein have been found in autistic subjects. In addition, an increased frequency of the extended haplotype B44-SC30-DR4, which contains a C4B null allele, has been reported in the same autistic group. In other studies, possible viral associations have been found
between autism and rubella or cytomegalovirus infections.\textsuperscript{13,14} Many autistic children frequently lacked rubella antibodies in spite of previous vaccinations.\textsuperscript{15} These investigations suggest something is awry in the immune system of autistic children. Therefore, an investigation of immune mechanisms and their possible relationship to autism seems worthwhile.
CHAPTER II
LITERATURE REVIEW

AUTISM

Autism is a developmental disorder that occurs at a frequency of two to five cases per 10,000 individuals with the rates of the disorder four to five times higher in males than females. An estimated 75% of autistic individuals function within a retarded range. The incidence of births of children with autism appears to be seasonal, peaking mostly in March. The existence of seasonal fluctuations suggests the involvement of an infectious agent, such as a virus, which often causes epidemics during winter months, as a potential etiological factor in some cases of autism. Persons diagnosed with autism appear to have normal life spans and normal physical appearance. Early diagnosis of autism is important because the child may begin treatment before his or her condition has deviated too far from normal. However, diagnosis of autism before the age of 12 months is difficult because no signs indicate the presence of autism during infancy. By definition, the onset of autism must occur before the child completes his or her third birthday. There are three types of behavioral symptoms observed in autistic children. These symptoms form the principal
criteria used in the diagnosis of autism and are classified as the following categories:

1. **Abnormal development of social relationships.**
   There is wide variation in the degree of expression of each behavioral symptom depending on the severity of the disorder and the age of the patient. Consequently, a symptom exhibited in one child may differ markedly in another. An autistic child encounters difficulty entering into and maintaining normal relationships with other people. He or she may treat people as inanimate objects, often unaware or unresponsive to feelings or emotions of others. For example, an autistic child's mother may be injured and in great pain. The child will observe the mother's reaction without any emotional response at all, as if he is bewildered and confused by the mother's conduct.

2. **Impairment of communication.** Many children with autism do not develop a useful form of speech (i.e. they do not produce sounds of recognizable words). Many of those who do achieve well developed speech have other speech abnormalities. These abnormalities may include echolalia in which the child repeats a word or phrase spoken by another person or uses neologisms, words that have a specific meaning not understood or used by others. Also, children with autism do not appear to use, to be aware of, or to interpret non-
verbal forms of communication such as facial expressions, gestures, eye-to-eye contact, etc.

3. **Child's interests and activities are restricted and repetitive.** Autistic children show to some degree an obsession with the maintenance of sameness. Variations of routine activities or environment may greatly disturb the child, who usually responds to the change with some sort of tantrum. For instance, one child has violent tantrums if his mother puts on a new dress, making it necessary for her to purchase many dresses of the same color and pattern so as to "keep the peace." Another autistic child makes straight long lines of bricks for hours, lining the bricks up in exactly the same way and in the same precise sequence of colors.

**GENETIC FACTORS IN AUTISM**

Bolton and Rutter have suggested that most cases of autism include some underlying genetic factor.23 This estimate is supported by two twin studies carried out in different countries, England and Norway. The English study24 found a 36% concordance rate for autism in monozygotic twins versus a 0% concordance rate in dizygotic twins. The second study25 reported an amazing 89% and 0% concordance rate for autism in monozygotic and dizygotic twins, respectively. These results strongly suggest that a significant genetic influence exists.
Ritvo et al.\textsuperscript{26} studied 46 families with multiple incidences of autism and found evidence for an autosomal recessive form of inheritance. The same workers later completed an epidemiological study\textsuperscript{27} that revealed a 215 times greater chance of a person having autism if he or she is a sibling of a child with autism. It is thus evident that the etiology of many cases of autism is genetically based.

**AUTISM AS AN AUTOIMMUNE DISORDER**

Efforts in understanding the etiology of autoimmune diseases have proven to be formidable. However, despite a lack of thorough understanding of this phenomenon, a genetic influence is suspected in autoimmunity. This suspicion is based on findings of multiple incidence of an autoimmune disease within a family. Interestingly, Money et al.\textsuperscript{28} reported a case of a child with autism and an autoimmune disease, Addison’s disease (this child also had moniliasis). Moreover, two older brothers exhibited various clinical conditions including hypoparathyroidism, Addison’s disease, moniliasis, and alopecia totalis. A third brother and parents were healthy and asymptomatic of any identifiable disease condition. The authors concluded that the autistic state might have been attributed to autoantibodies in some way affecting the central nervous system.\textsuperscript{28}
Other autoimmune disorders including systemic lupus erythematosus and rheumatoid arthritis have been found in families of autistic children. Autoantibodies to brain serotonin receptors in autistic subjects have also been reported. In other studies, evidence has been found for immune reactivity to neural-tissue. These include studies performed by Weizman et al. and Singh et al., in which there was a lymphocyte response and formation of autoantibodies, respectively, to human myelin basic protein. Moreover, Plioplys et al. found the presence of autoantibodies to a different brain antigen, the 210K neurofilament. These same authors showed an abnormally increased percentage of DR-positive T-cells lacking IL-2 receptors in 17 autistic patients. This finding of DR-positive T-cells lacking IL-2 receptors in autistic patients was confirmed by Warren et al. and is a characteristic seen in autoimmune diseases.

**COMPLEMENT**

The complement activation system is one of many immune defense mechanisms that provides protection against pathogens via two pathways: the classical pathway and alterative pathway. Both pathways function in the extracellular fluid. The classical pathway consists of nine proteins that participate in a sequential cascade of reactions. C1, the
first protein of the pathway, is activated by an antibody bound to a membrane surface. The actual sequence for the classical complement components is C1-C4-C2-C3-C5-C6-C7-C8-C9 (see Figure 1). The first five components have enzymatic activity and are considered to be proenzymes (precursors of inactive form of enzyme). The last four form a membrane attack complex (MAC). This complex of proteins, the MAC, aggregates to form a hollow cylinder that is inserted into the membrane of the pathogen. This cylindrical complex functions by allowing cellular material to "leak" out and, more importantly, water to enter. The osmotic pressure causes cell lysis, destroying the pathogen.

The alternative pathway differs from the classical pathway in one fundamental way: how the pathway is initiated. The alternative pathway is triggered by C3 spontaneously attaching to a membrane surface, to microbial agents, to IgA, or to IgG4. Again, a sequence of reactions takes place with additional complement components Factor B, Properdin, and Factor D. The remaining components of this pathway, C5 through C9, are the same as for the classical pathway with the same result of cell lysis. Thus, the latter stages of each pathway are shared between both the classical and alternative pathways. The alternative pathway is part of the innate immune system that is nonspecific and active at all times.
C4A and C4B, the two major isotypes of C4, are encoded by two closely linked genes within the MHC. The two genes are nearly 98% homologous, and extensive polymorphism is observed at their respective loci. Each C4 locus produces
one large peptide chain that undergoes posttranslation modifications, such as internal cleavage and the formation of disulfide bonds to yield tripeptide molecules. The three subunits of the molecule have been labeled alpha, beta, and gamma, the alpha being the largest and the gamma chain being the smallest.

When a C4 molecule is activated (cleaved by C1s), a small fragment of the amino end of the a chain is cleaved off. Removal of this small fragment exposes two activation sites. One is involved in the binding complement protein C2, and the other is an unstable internal thioester bond. The latter is responsible for attaching the C4 molecule to a membrane (or antigen) surface and for the unique functional differences observed between C4A and C4B molecules. C4A appears to preferentially bind to amino-rich surfaces by forming amide linkages via hydrolysis of the thioester bond; C4B tends to form ester linkages from hydrolysis of the same bond. This difference in avidity to distinct functional groups is believed to be responsible for the unique functions of C4A and C4B. For example, C4B is four times more hemolytic to sheep red-blood cells than C4A. This is likely due to a predominance of hydroxyl groups on the red blood-cell surface and therefore the greater deposition of C4B on the membrane. Other functional differences between C4A and C4B may be similarly explained.
FUNCTIONAL ROLE OF COMPLEMENT

Although complement provides a defense mechanism by causing cell lysis, additional protection is accomplished through a process termed opsonization. Opsonization is a process in which foreign particles are "marked" for cellular uptake by fragments of complement components produced by enzymatic cleavage during activation of the complement pathways. The C3b and C4b complement fragments are referred to as opsonins, and when deposited on a cell surface act as ligands for phagocytic cells. This mechanism provides enhanced phagocytic efficiency. Therefore, the clearance and elimination of potential pathogenic organisms are increased via opsonization.

C4B NULL ALLELE AND AUTISM

One of the primary objectives of this project is to provide data to suggest a possible association of a C4B null allele with autism. A null allele is a an allotype that does not produce a functional gene product. The null allele may not produce any gene product at all or may produce an inactive gene product. In earlier studies it is suggested that autistic patients have a higher incidence of a null allele at the C4B locus than patients without the disorder.12
The presence of a C4B null allele constitutes a complement-component deficiency and results in decreased serum levels of C4B protein. Low levels of any complement component may limit the defensive mechanism provided by the complement activation system.

EXTENDED MHC HAPLOTYPES

The major histocompatibility complex (MHC) is located on the short arm of human chromosome 6. Within this region of about three million base pairs lie many genes involved in the immune system (see Figure 2). The combination of all alleles within the MHC determines the haplotype for the chromosome observed. Therefore, a person will normally have two haplotypes (one for each chromosome 6). These genes have been categorized into three classes: I, II, III. From the centromere the MHC gene classes are arranged in the following order: MHC II-MHC III-MHC I. Most MHC I and MHC II gene products are cell surface proteins. However, MHC III proteins are not directly associated with a cell membrane. MHC I gene functions involve processing and presentation of antigens (small peptides) to T-cells, primarily CD8+ T-cells. While MHC II genes also function in antigen processing and presentation, their interaction is primarily restricted to CD4+ T-cells. MHC III genes code for a variety of products and have a wide array of functions, including cytokine
signaling and complement function. The three class III genes studied in this project are C4A, C4B, and Bf.

DR  C4B  C4B  C2  BF  B
Centromere --|---//---|------|------|------|---//---|--- Telomere

Fig. 2. Simple schematic of the MHC on chromosome 6.

An extended MHC haplotype is a fixed conserved region within the MHC usually defined by HLA-B (MHC II), compleotype Bf,C2,C4A,C4B, and HLA-DR (MHC II) alleles. However, the conserved region may extend beyond these genes. Because these genes behave as if they are "fixed," they are inherited as a group, showing a lower frequency of crossing-over than expected. This phenomenon is referred to as linkage disequilibrium.

MHC-DISEASE ASSOCIATION

Linkage disequilibrium is one of two distinguishing features of the MHC. The other is extensive genetic polymorphism. Extensive polymorphism is a possible selective advantage for survival and, conversely, there is a possibility of association of certain diseases with particular MHC profiles (HLA profile), or haplotypes. Many such associations have been identified. For example, it has been found that a person whose HLA profile includes HLA-B27
has an 87.4 times greater risk of developing Ankylosing spondylitis than a person without HLA-B27. This is a strong association. The most common extended haplotype studied to date is designated B8-SC01-DR3 and has been found to be associated with several disease conditions including generalized myasthenia gravis, IgA deficiency, and systemic lupus erythematosus.

The mechanism responsible for the association of a particular MHC profile and disease remains unclear. However, the MHC's central role in processing and presenting foreign antigens for elicitation of an immune response is a possible explanation. One possibility is that the MHC proteins are not able to adequately process a pathogen and thereby activate necessary mechanisms for a host to mount an immune response. This inability of the host to defend itself immunologically would allow the pathogen to cause sufficient damage to result in disease.

**MHC CLASS II HYPERVARIABLE REGIONS**

Of all human genes known, MHC genes are thought to exhibit the highest degree of allelic polymorphism. The MHC Class II complex consists of two peptide subunits termed alpha and beta. Together, the two subunits fold to form an "antigen binding groove" where fragments of foreign proteins, 6 to 25 amino acids long, are loaded following degradation.
processes. These degraded fragments then act as antigens when presented in conjunction with MHC molecules to T-cell receptors. Most T-cells can only respond to antigens presented in this fashion. This is the commonly known phenomenon of MHC restriction.

Interestingly, in allelic variants of HLA-DR molecules, nearly all the polymorphisms are contributed by the beta subunit, most of which lie in three areas termed the hypervariable regions (HVRs). Correlation of polymorphic sites to the tertiary structure of the HLA-DR molecule is evident of an assignment of the hypervariable regions to the antigen-binding groove of the MHC complex. This implies that the antigenic fragment lying within the antigen-binding groove is in direct, or at least close, contact with the HVRs of the MHC subunits. The HVRs provide a crucial portion of a framework for the selective interaction of MHC molecules with antigenic peptides. One antigenic peptide may bind to the antigen-binding groove with greater affinity than another peptide. Such selectivity determined by the HVR specificities of the HLA molecules is the essence of the strength and efficiency of an immune response.

HLA-DR allotypes can be further subtyped through analysis of amino acid sequences coded by the DRB1 gene. More specifically, the heterogeneity of the HLA-DR4 subtypes can be traced to and identified by amino acid differences at
positions 37, 57, 67, 70, 71, 74, and 86 of the beta subunit. Based on sequence specificities, the subtypes are designated DRB1*0401, DRB1*0402, DRB1*0403, etc. The variations between subtypes may exist at one, two, or all three HVRs.

Several research groups are studying potential roles HVR specificities may play in disease pathogenesis. Matsushita et al. noted that patients with methimazole-induced insulin autoimmune syndrome express the DRB1*0406 allele and not DRB1*0405 allele. The group performed binding assays in which a small peptide fragment of human insulin alpha chain bound with much greater affinity to DRB1*0406 than to DRB1*0405. The authors conclude that presentation of the small fragment by antigen-presenting cells may lead to activation of self-insulin-specific T-helper cells. Another study performed by Topham et al. looked at experimental autoimmune encephalomyelitis (EAE), which represents a mouse model for human multiple sclerosis. Mice were vaccinated with an 18-amino acid peptide from HVR3 of the mouse MHC class II beta chain. The treatment elicited auto-antibodies to the small beta peptide and aided in preventing and treating EAE. A similar avenue may be useful in the treatment of human autoimmune diseases associated with specific HVR sequences.
CHAPTER III
MATERIALS AND METHODS

SUBJECTS

One hundred twenty-four subjects were included in this study; 45 autistic child subjects, 30 normal child subjects, and where possible, the parents of each participating child were also analyzed by the same methods. Diagnosis of infantile autism was made by using DSM-IIIR criteria as established by at least two psychiatrists or psychologists. All subjects were of Northern European descent and were living in the same geographical area (Northern Utah) at the time of this study. In addition, all normal subjects were screened for possible autoimmune disorders (e.g., adolescent diabetes).

PLASMA PREPARATION

Blood samples used in this study were collected via venipuncture following informed consent procedures. Freshly drawn samples were centrifuged at 2,000 rpm (900x g) for 10 minutes. Plasma was stored at -70°C until used.

ELECTROPHORESIS OF C4A AND C4B

Prior to electrophoresis, plasma samples were treated with two enzymes, Neuraminidase Type V and Carboxypeptidase B
Type II (both from Sigma, St. Louis, MO). Approximately 0.13 units of neuraminidase were added to 10 µl of plasma and incubated for 18 hours at 4°C followed by 1 hour of incubation at 37°C. Approximately 1 to 1.3 units of carboxypeptidase B were added to each sample and incubated for 30 minutes at room temperature (≈25°C).

Agarose gels (SeaKem ME Agarose, FMC) were prepared with electrophoresis buffer (8 mM Barbital, 32 mM Sodium Barbital, 37 mM Glycine, and 19 mM Tris[hydroxymethyl]aminomethane) and distilled H₂O. Approximately 13 ml of liquified 0.7% agarose was loaded on the hydrophilic side of a 10-cm x 10-cm electrophoresis film (i.e. gel bond) to create a 1.3-mm thick gel. Sample wells were formed in the gel with the use of a 15-well, 0.75-mm comb (BioRad, Hercules, CA). The gel was allowed to cool and solidify.

The gel was placed on a cooling plate (Horizontal Electrophoresis Cell, BioRad, Hercules, CA) at 4°C with the wells near the cathode. Wicks were placed at both ends of the gel so as to bridge the buffer chambers and the gel. Four microliters of treated samples were loaded into each well. The same volume of sickle cell hemoglobin (HbS, Sigma, St. Louis, MO) was loaded in the two outermost wells. Electrophoresis was terminated when the HbS markers migrated 7 cm anodally (about 3 hours). Electrophoresis was performed
at 400 constant volts while limiting the current at 200 mA and the power at 200 W.

IMMUNOFIXATION AND STAINING OF C4

Following electrophoresis, the gel was removed from the refrigerated plate, and 0.5 ml of a 3:2 dilution of anti-human C4 antibody and distilled water (Atlantic Antibodies, Stillwater, MN) was spread on the surface of the gel (about 5μl/cm²). The gel was placed in a humidified box for 2 hours at room temperature. The antibody diffused into the gel and bound to C4 present to form a large antigen-antibody complex. This process is referred to as immunofixation because the antigen-antibody complex becomes insoluble and is immobilized within the gel.

When immunofixation was complete, filter paper (Whatman No.5) was placed over the gel with light pressure to absorb excess or unbound antibody. The gel was then placed in 1X phosphate buffered saline solution (PBS, 1.9 mM sodium phosphate monobasic, 8.1 mM sodium phosphate dibasic, 154 mM sodium chloride, pH 7.4) overnight so any protein not bound by the anti-human C4 antibody could diffuse out of the gel. Therefore, only the complexed C4 protein remained in the gel. The next day the PBS was replaced with fresh PBS twice, one hour for each change.
Next, the gel was dried with a hair dryer. The dried gel was placed in a staining solution (1% Coomassie Brilliant Blue G-250, J.T. Baker) for 10 minutes, then in a destaining solution until the background was clear compared to the stained bands. The gel was preserved by rinsing in a 10% glycerol solution and dried again using a hair dryer. At this point blue "spots," or bands, may be visualized within the gel. These bands represented various C4 alleles and were used for designating specific C4 allotypes for each patient.

**C4 ALLOTYPING**

C4 allotyping, or complotyping, is based on the relative migration rates of C4A and C4B. C4A3 and C4B1 are the most common C4 allotypes in humans. The distance between these two bands is taken as a standard of unity. Where a band is located relative to the band positions of C4A3 and C4B1 determines its designated allotype. The appearance of a single faded or weak band strongly suggests the presence of a C4 null allele. The presence of a null allele can be supported by a corresponding faded band in the plasma of one of the patient's parents. Also, the patient's measured C4 protein serum level is expected to be low or moderately low. A patient with one C4B null is considered heterozygous for the C4B gene. If no C4B bands are visualized, the patient
likely has two C4B null alleles and is considered homozygous for the gene.

**PREPARATION OF CELLS FOR DNA EXTRACTION**

Ten milliliters of venous whole blood were layered over 4 ml Histopaque 1077 (Sigma, St. Louis MO) and centrifuged for 20 minutes at 1,000x g. Human peripheral blood mononuclear cells (PBMC), located at the top of the Histopaque solution (i.e. at the interface), were transferred to a 15-ml conical bottomed tube, and the volume was brought to 10 ml with RPMI-1640 (Gibco Lab, Grand Island, NY). The tube was centrifuged at 500x g for 10 minutes. The pellet was then resuspended in 5 ml RPMI-1640 and centrifuged a second time at 200x g for 5 minutes. Finally, the pellet was resuspended in 5 ml RPMI-1640 and 3 x 10^6 PBMCs were transferred into a 25-cm² tissue culture flask containing 10 ml HyQ Cyte L (HyClone Lab, Logan UT) and 1% phytohemagglutinin (PHA) (Gibco Lab, Grand Island, NY). The flask was incubated for 5 days at 37°C.

**EXTRACTION OF DNA FOR FACTOR B TYPING**

After a patient’s PBM’s had proliferated to a concentration of 10^6 to 10^7 cells/ml (see above), the cultured solution was centrifuged at 2,000 rpm (900x g) for 10
minutes. The supernatant was removed and 240 μl of lysis buffer (0.28 M sucrose, 10 mM Tris-HCL at pH 7.5, 5 mM MgCl₂ and 1% Triton X-100) and 18 μl of Pronase E (20 mg/ml, Sigma, St. Louis, MO) were added to the pellet and mixed well. This mixed solution was transferred to a microtube.

Twelve microliters of 20% sodium dodecyl sulfate were added and the mixture was incubated at 55°C for 12 to 16 hours (overnight) while shaking gently. An equal volume of phenol was added to the incubated mixture and vortexed for 15 minutes followed by centrifugation, 14,000 rpm, for 10 minutes. The aqueous layer was transferred to a separate microtube. Again, an equal volume of phenol was added followed by vortexing, centrifugation, and transfer of aqueous phase as mentioned above. An equal volume of 25:1 chloroform:isoamyl alcohol was added to the transferred aqueous solution and vortexed for 15 minutes followed by centrifugation in a microcentrifuge at 14,000 rpm (6,000x g) for 10 minutes. The aqueous phase (top layer) was transferred to a separate microtube.

The DNA was precipitated by adding 0.1X volume of 3 M sodium acetate and 1.5X volume of isopropanol. The precipitated DNA was spooled onto the tip of a pasteur pipet and placed in 0.5 ml TE buffer (10 mM Tris, 1 mM EDTA) to dissolve the DNA. A 0.5X volume of 7.5 M ammonium acetate and 2X volumes ice-cold ethanol (100%) were added to the DNA
to reprecipitate it. The DNA was spooled and placed into a fresh microtube. The ethanol was allowed to evaporate, and 200 µl TE-4 buffer (10 nM Tris, 0.1 mM EDTA) was added to the sample. The DNA was dissolved overnight. DNA concentration was determined spectrophotometrically at O.D. 260 nm and a measure of DNA purity was determined by obtaining a 260/280 ratio.

METHODS FOR TYPING FACTOR B

The two methods used for allotyping of the Factor B gene (Bf gene) were polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). For PCR, a 50-µl solution was prepared with the following reactants: patient’s DNA (1 µg/ml), 1.5 mM MgCl₂, 200 µM deoxynucleotide phosphate bases, 1X PCR buffer (Promega Corp., Madison, WI), 1 µM of each primer (5’AAGTGATGTGGGTAGGACAGGC3’ and 5’TGCAGTCTGCCTTCCTGACAGGAC3’), and 2 units of Taq DNA Polymerase (Promega Corp., Madison, WI). Finally, an equal volume of mineral oil was placed on top of the prepared solution to prevent evaporation. PCR was carried out for 30 cycles in an Ericomp thermal cycler. Each cycle consisted of a 1-minute denaturation at 94°C, a 2-minute annealing at 64°C, and 2-minute extension at 72°C. A 593-bp oligonucleotide PCR product was detected by agarose gel electrophoresis. Four microliters of the PCR product and 2
μl of tracking dye (50% glycerol, 0.25% bromophenol blue, 10 mM EDTA) were combined and loaded onto a 2% NuSieve Agarose (FMC Bioproducts, Rockland, ME) gel in a 1X Tris acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA, 0.5 μg/ml ethidium bromide). A DNA size marker (1 Kb DNA ladder, Gibco BRL, Gaithersburg, MD) was loaded in the gel to estimate the approximate the size of the PCR product and ensure proper amplification of desired DNA region. The gel was run at 100 mA for approximately 40 minutes. The gel was placed on a UV light transilluminator and the DNA was detected by fluorescence of ethidium bromide. Photographs of each gel were taken for ease of storing collected data.

Samples with positive PCR amplification were analyzed for the MspI RFLP of the BF gene. The PCR product was digested with the isoschizomers MspI or HpaII (Promega Corp., Madison, WI) in the following manner. A 30-μl reaction solution was prepared by combining 6 μl of the amplified product, 3 μl 10X buffer for enzyme, 3 units of endonuclease, and 20.7 μl sterilized dH2O. The solution was vortexed gently and placed in a 37°C waterbath overnight. Next, 10 μl of tracking dye were added to each sample and loaded in a 4% NuSieve Agarose gel. The gel was loaded with 40 μl of sample per well. Again, a DNA size marker (1Kb DNA ladder, Gibco BRL) was also loaded along with samples. The buffer used was
a 1X Tris-acetate-EDTA buffer with ethidium bromide. Electrophoresis ran for 50 minutes limited at 100 mA.

The DNA fragments were visualized on a transilluminator and photographs taken. The S allotype of the Bf gene contains an MspI site that produces two fragments after digestion of approximately 400 and 200 bp. An uncut 600 bp fragment depicted an F allotype of the Bf gene.
CHAPTER IV
RESULTS

C4 ANALYSIS

In Figure 3, an immunoelectrophoresis gel of treated plasma samples is depicted. The dark uppermost and lowermost bands are the reference C4A and C4B allotypes, respectively, which serve as reference points for C4 allotyping. The uppermost band is C4A3 and the lowermost band is C4B1. The distance between C4A3 and C4B1 is given a reference value of 100. Where other C4 allotypes migrate with respect to C4A3 and C4B1 determines its designated phenotype. Figure 4 is a RFLP electrophoresis gel for Factor B. Lane 1 is a DNA size marker. In lane 2 there is an endonuclease cut site on one chromosome, represented the 200bp and 400bp bands (S allotype), and the lack of the same endonuclease cut site on the other chromosome, represented by the 600bp band 9F allotype). In lane 3 there is only the 600bp band (therefore homozygous F), while in lane 4 there are the 400bp and 200bp bands, but not the 600bp band (therefore homozygous S).

At least one C4B null allele was found in 22 of 45 (49%) autistic subjects (Figure 5). However, only 16 (20%) of the 79 normal subjects carried the same allele. This increase in the number of C4B null alleles found in the autistic subjects was statistically significant (p <0.001). Of the 21 autistic
**Fig. 3.** Gel analysis of C4A and C4B phenotypes.

**Fig. 4.** RFLP analysis of Factor B.
Fig. 5. Percentage of autistic subjects, their parents, and unrelated healthy controls expressing a C4B null allele.
patients who did carry a C4B null allele, 8 carried an additional C4 null allele, C4A or C4B, on the homologous chromosome. A C4A null allele was carried on 20 of 45 (44%) of the autistic subjects and on 37 of 79 (47%) of the normal subjects. This was not found to be significant statistically. Although the importance is not known, two C4 null alleles were never found to be carried on the same chromosome.

The percentages of C4B and C4A null alleles of the parents of the autistic patients and controls are included in Figures 5 and 6, respectively. The purpose of including the parental data was to see if perhaps C4 null alleles were inherited more maternally or paternally. As can be observed from Figures 5 and 6, no such tendency is evident. However, if a child expressed a null allele, at least one parent also carried a null allele.

The C4 allotypes of patients, autistic and nonautistic, carrying a C4B null allele are given in Table 1. No one allele was found to be significantly associated with a C4B null allele in the presence of autism.

EXTENDED HAPLOTYPE ANALYSIS

Ten different extended haplotypes (Table 2) were observed in this study of autistic and normal subjects randomly selected from the general Northern Utah population.
Fig. 6. Percentage of autistic subjects, their parents, and unrelated healthy controls expressing a C4A null allele.
### Table 1. C4 allotypes in patients with C4B null alleles

<table>
<thead>
<tr>
<th></th>
<th>Autistics (n = 21)</th>
<th>Nonautistics (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 1 2 3 4 5 6</td>
<td>0 1 2 3 4 5 6</td>
<td></td>
</tr>
<tr>
<td>C4A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3 21</td>
<td>1 4 1</td>
</tr>
<tr>
<td>C4A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7 11 1 2</td>
<td>1 4 1</td>
</tr>
<tr>
<td>C4B&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1 18 1 1</td>
<td>2 4</td>
</tr>
</tbody>
</table>

- <sup>a</sup>This is the C4A allotype found on the same chromosome as the C4B null allele.
- <sup>b</sup>These are the C4 allotypes found on the chromosome not carrying the C4B null allele.
Table 2. List of extended haplotypes observed in this study

<table>
<thead>
<tr>
<th>Class I</th>
<th>Class III</th>
<th>Class II</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>Bf</td>
<td>C2</td>
<td>C4A</td>
</tr>
<tr>
<td>7</td>
<td>S</td>
<td>C</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>S</td>
<td>C</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>S</td>
<td>C</td>
<td>3</td>
</tr>
<tr>
<td>18</td>
<td>F1</td>
<td>C</td>
<td>3</td>
</tr>
<tr>
<td>35</td>
<td>F</td>
<td>C</td>
<td>3,2(^a)</td>
</tr>
<tr>
<td>44</td>
<td>S</td>
<td>C</td>
<td>3</td>
</tr>
<tr>
<td>44</td>
<td>F</td>
<td>C</td>
<td>3</td>
</tr>
<tr>
<td>57</td>
<td>S</td>
<td>C</td>
<td>6</td>
</tr>
<tr>
<td>62</td>
<td>S</td>
<td>C</td>
<td>3</td>
</tr>
<tr>
<td>65</td>
<td>S</td>
<td>C</td>
<td>2</td>
</tr>
</tbody>
</table>

\(^a\) This designation denotes a rare duplication of the C4A gene in which one allele is expressed as C4A3 and the other allele as C4A2. With the extended haplotype B35-FC(3,2)-DR1 it has been shown that the gene duplication results from gene conversion of B to A. The extended haplotype B44-SC30-DR4 has undergone a similar conversion but it is conventionally designated as B44-SC30-DR4 instead of B44-SC(3,3)0-DR4.
These 10 extended haplotypes were expressed on 53 of 90 (58.8%) (Table 3) chromosomes studied in the autistic patients. The same extended haplotypes were carried on only 33 of 128 (25.7%) chromosomes of unrelated normal subjects. Also, mothers of the autistic children had an increased representation of extended haplotypes, 41 of 78, compared to that of the fathers, 31 of 78 (Table 3). Many extended haplotypes have been found to be associated with various diseases. Diseases known or suspected to be associated with the extended haplotypes found in this study are displayed in Table 4.

The phenotypic frequencies of the extended haplotypes observed in this study are shown in Table 3. Of the 10 extended haplotypes found in this study, only one B44-SC30-DR4 was significantly increased (p < 0.05). B44-SC30-DR4 was not represented in any of the 64 control subjects.

In addition to the significant representation of B44-SC30-DR4, four autistic subjects had a fragment of this extended haplotype including SC30-DR4 (Table 5) (i.e. the fragment SC30-DR4 includes the Class II and Class III regions of this extended haplotype and a different Class I region allele). Thus, the extended haplotype B44-SC30-DR4, or a fragment thereof, is represented in nearly a third (29%) of the autistic subjects as compared to less than 2% of the unrelated healthy controls. Moreover, 12 mothers of autistic
Table 3. Frequency of extended MHC haplotypes in autistic subjects

<table>
<thead>
<tr>
<th>Ext. Haplotype</th>
<th>Total</th>
<th>Maternal</th>
<th>Paternal</th>
<th>Healthy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 45</td>
<td>n = 39</td>
<td>n = 39</td>
<td>n = 64</td>
</tr>
<tr>
<td>B44-SC30-DR4</td>
<td>9 (20.0)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8 (20.5)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3 (7.7)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>BX-SC30-DR4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4 (8.8)</td>
<td>4 (10.3)</td>
<td>2 (5.1)</td>
<td>1 (1.6)</td>
</tr>
<tr>
<td>B35-FC(3,2)0-DR1</td>
<td>2 (4.4)</td>
<td>0 (0)</td>
<td>3 (7.7)</td>
<td>1 (1.6)</td>
</tr>
<tr>
<td>B18-F1C30-DR3</td>
<td>2 (4.4)</td>
<td>1 (2.6)</td>
<td>1 (2.6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>B62-SC33-DR4</td>
<td>2 (4.4)</td>
<td>2 (5.1)</td>
<td>2 (5.1)</td>
<td>2 (3.1)</td>
</tr>
<tr>
<td>B44-FC31-DR7</td>
<td>7 (15.5)</td>
<td>4 (10.3)</td>
<td>4 (10.3)</td>
<td>4 (6.2)</td>
</tr>
<tr>
<td>B57-SC61-DR7</td>
<td>3 (6.7)</td>
<td>4 (10.3)</td>
<td>1 (2.6)</td>
<td>2 (3.1)</td>
</tr>
<tr>
<td>B13-SC31-DR7</td>
<td>3 (6.7)</td>
<td>2 (5.1)</td>
<td>1 (2.6)</td>
<td>1 (1.6)</td>
</tr>
<tr>
<td>B65-SC2(1,2)-DR1</td>
<td>1 (2.2)</td>
<td>1 (2.6)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>B7-SC31-DR15</td>
<td>7 (15.5)</td>
<td>6 (15.4)</td>
<td>6 (15.4)</td>
<td>5 (7.8)</td>
</tr>
<tr>
<td>B8-SC01-DR3</td>
<td>13 (28.8)</td>
<td>9 (23.1)</td>
<td>8 (20.5)</td>
<td>17 (26.6)</td>
</tr>
</tbody>
</table>

<sup>a</sup> p <0.001 as compared to values from healthy controls.

<sup>b</sup> Fragment of the B44-SC30-DR4 extended haplotype including SC30 and DR4 but with a HLA B marker other than B44.
Table 4. List of extended haplotypes observed in this study and their reported disease associations\textsuperscript{49}

<table>
<thead>
<tr>
<th>Extended Haplotype</th>
<th>Disease Associations</th>
</tr>
</thead>
<tbody>
<tr>
<td>B7-SC31-DR15</td>
<td>Multiple sclerosis, IgA deficiency</td>
</tr>
<tr>
<td>B8-SC01-DR3</td>
<td>Generalized myasthenia gravis, IgA deficiency, Systemic lupus erythematosus, Celiac disease</td>
</tr>
<tr>
<td>B13-SC31-DR7</td>
<td>Insulin-dependent diabetes mellitus</td>
</tr>
<tr>
<td>B18-F1C30-DR3</td>
<td>HIV rapid progression</td>
</tr>
<tr>
<td>B35-FC(3,2)-DR1</td>
<td>Rheumatoid Arthritis (child onset), Feltys Syndrome</td>
</tr>
<tr>
<td>B44-SC30-DR4</td>
<td>Celiac disease, IgA deficiency</td>
</tr>
<tr>
<td>B44-FC31-DR7</td>
<td>IgA deficiency, Psoriasis</td>
</tr>
<tr>
<td>B57-SC61-DR7</td>
<td>Insulin-dependent diabetes mellitus, Rheumatoid Arthritis</td>
</tr>
<tr>
<td>B65-SC2(1,2)-SR1</td>
<td>IgA deficiency</td>
</tr>
<tr>
<td>Subjects</td>
<td>N</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>----</td>
</tr>
<tr>
<td>Autistics</td>
<td>45</td>
</tr>
<tr>
<td>Mothers</td>
<td>39</td>
</tr>
<tr>
<td>Fathers</td>
<td>39</td>
</tr>
<tr>
<td>Family normal chromosomes</td>
<td>39</td>
</tr>
<tr>
<td>Unrelated controls</td>
<td>64</td>
</tr>
</tbody>
</table>

*a* Fragment of B44-SC30-DR4 with identical DR, DQ and complement alleles but with a different B region allele.

*b* \( p < 0.001 \) as compared to unrelated controls.

*c* \( p < 0.05 \) as compared to unrelated controls.

d Chromosomes of parents not segregating to autistic child.
children carried B44-SC30-DR4 or its fragment. In all, 18 of
45 (40%) of the autistic subjects or their mothers carried
B44-SC30-DR4 or its fragment.

Seven of the autistic subjects in this study had one or
more siblings also diagnosed with autism. Their extended
haplotypes and those of their siblings are given in Table 6.
Interestingly, five of these subjects carried an extended
haplotype on both chromosomes, and a sixth subject carried an
extended haplotype on one chromosome. Five autistic siblings
or sets of autistic siblings shared one or both extended
haplotypes expressed by the corresponding reference autistic
subjects. One autistic subject (Subject 6 in Table 6)
carried an extended haplotype not received by the autistic
sibling, and one autistic subject (Subject 7) and sibling did
not express any extended haplotypes at all.
Table 6. Sharing of extended haplotypes by autistic siblings

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sibling 1</th>
<th>Sibling 2</th>
<th>Sibling 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. B8-SC01-DR3</td>
<td>B8-SC01-DR3</td>
<td>B8-SC01-DR3</td>
<td>B8-SC01-DR3*</td>
</tr>
<tr>
<td>B13-SC31-DR7</td>
<td>B13-SC31-DR7</td>
<td>B13-SC31-DR7</td>
<td>None</td>
</tr>
<tr>
<td>2. BX-SC30-DR4</td>
<td>BX-SC30-DR4</td>
<td>B8-SC01-DR3</td>
<td>B8-SC01-DR3</td>
</tr>
<tr>
<td>B7-SC31-DR15</td>
<td>None</td>
<td>B7-SC31-DR15</td>
<td>B7-SC31-DR15</td>
</tr>
<tr>
<td>3. B44-SC30-DR4</td>
<td>B44-SC30-DR4</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>B57-SC61-DR7</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>4. B35-SC(3,2)0-DR1</td>
<td>B35-SC(3,2)0-DR1</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>B44-FC31-DR7</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>5. B62-SC33-DR4</td>
<td>B62-SC33-DR4</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>B44-FC31-DR7</td>
<td>B8-SC01-DR3</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>6. B44-FC31-DR7</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>None*</td>
<td>None*</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

*a Subject 1 had a fourth sibling who also inherited B8-SC01-DR3 and whose other chromosome did not express an extended haplotype.

b Chromosome carries B62-SC31-DR15-DRW52-DQ6, which is a fragment of B7-SC31-DR15 (Table 1).
CHAPTER V
DISCUSSION

Two significant findings of this study include increased frequencies of the C4B null allele and the extended haplotype B44-SC30-DR4 among autistic patients. The actual relationship of either finding to autism is unknown, although numerous mechanisms can be imagined. One possibility is that the extended haplotype B44-SC30-DR4 carries a functionally silent C4B null allele. The products of the C4 genes are essential to the activation of other critical components of complement pathways that provide protection against viruses, bacteria, and other pathogens. Increased frequency of C4B null alleles has been reported in patients with scleroderma and schizophrenia. In addition, accelerated disease progression following seroconversion to HIV and certain manifestations associated with AIDS have been associated with C4 null alleles. C4B null alleles have also been found to be underrepresented in people 80 years of age or older. This underrepresentation implies that expression of a C4B null allele may have a deleterious effect on a person's long-term survival. It is logical to conclude that a person without the complement defense mechanism may have a greater predisposition to disease by infectious agents.
A small subset of autistic patients in this study did not carry a C4 null allele but their mothers did. At first glance this may argue against an association of a C4 deficiency with autism. However, it is plausible, in some cases, that the mother's immune deficiency (i.e. having a C4 null allele) may be sufficient to allow a pathogen to persist and cause damage to the fetus during gestation. Interestingly, Laitinen et al. found spontaneous abortions to be associated with C4 null alleles.

The relevance of B44-SC30-DR4 to autism may be through the Class II markers expressed on this extended haplotype. This study found the Class II and III portions (-SC30-DR4) of B44-SC30-SR4 in 18 of 45 (40%) autistic subjects and/or autistic mothers. The Class II (DR and DQ) markers are intimately involved in binding and presenting degraded processed peptide antigens to T lymphocytes. Class II markers, with a peptide antigen ligand, interact with T-cell receptor (TCR) molecules, resulting in activation of the mature T lymphocyte. If the Class II-TCR peptide complex interaction occurs during maturation of the T cell in the thymus, two types of selection will take place: positive and negative selection. These selection processes allow T-cells to differentiate into competent mature T lymphocytes. During intrathymic development, negative selection appears to eliminate T-cells specific for self peptides due to their
potentially harmful effects. In the positive selection process, T-cell receptors that interact with self MHC molecules with appropriate affinity evade cell death. All other developing T-cells die. Both processes are necessary to acquire T-cell maturity, and both are intrinsic in creating a T-cell repertoire that is unique to an MHC haplotype of an individual.

Both the antigen selection and binding function of MHC molecules are potential mechanisms that may explain HLA disease associations. The specific role of either mechanism to any disease remains unknown. However, several models have been proposed to explain disease associations to MHC haplotypes. In the first model, it is suggested that the MHC molecule itself operates as a receptor for the pathogen or disease-causing agent. Therefore, the MHC specificity (i.e. an individual’s haplotype) of an individual would determine a pathogen’s ability to infect a host and cause disease. In the second model, it is proposed that antigenic fragments from foreign agents (or self peptides in the case of autoimmune diseases) are selectively bound, with extreme efficiency, into the antigen binding groove of a particular MHC molecule. Hence, only persons expressing that specific MHC molecule would be able to mount an immune response to that specific antigenic determinant of the disease-causing agent.
In another hypothesis it is postulated that MHC-associated diseases arise from molecular mimicry that exists between the MHC molecule and the pathogenic agent.\textsuperscript{59,60} In this model it is suggested that antigenic similarities occur or are shared between an etiological agent and the MHC disease associated molecule. Such similarities could result in an infection unchallenged because the host would lack those clones specific for MHC self-peptides that were eliminated during negative selection. In other words, the host would not have any T-cells able to recognize the infectious agent. Alternatively, if MHC-derived peptides were not utilized during negative selection, antigenic similarities could provoke a strong immune response to self tissues causing considerable tissue damage. This model, in which antigenic similarities between a pathogen and MHC molecules are proposed, is also known as the shared epitope hypothesis.\textsuperscript{61,62} In several studies molecular mimicry in disease states have been implicated.\textsuperscript{63}

Finally, in a fourth hypothesis it is proposed that it is the T-cell repertoire determined by the MHC profile of an individual that is critical to the pathogenesis of a disease. Certain TCR specificities may be necessary to induce pathogenesis of a particular disease. These required specificities may only be present in an individual with a specific HLA allotype.
A number of research groups are studying the hypervariable regions (HVRs) of HLA molecules in an effort to link specific sequences within these areas to disease states. The MHC antigen subtypes DRB1*0401 and DRB1*0101 have been associated with rheumatoid arthritis (RA). For RA, it is believed that the basis for such associations is the existence of an amino acid sequence that is common between the third HVR of the beta chain of the RA associated MHC alleles and an epitope of an infectious agent. For example, one study of rheumatoid arthritis patients found cross-reactivity between the Escherichia coli heat shock protein dnaJ and the DRB1*0401 molecule. The cross-reactivity suggested an epitope shared by both proteins. Another study, also including rheumatoid arthritis patients, determined the existence of a highly homologous sequence between a DR4 molecule and an Epstein-Barr virus glycoprotein. In both cases, the sequence displaying homogeneity was located in the third hypervariable region of the HLA molecule. Exactly how MHC molecules affect susceptibility to RA is not known; however, the HVRs of the MHC molecules may control the binding of a arthritogenic peptide.

Peptides from the third hypervariable region of MHC DR4 alleles generally elicit immune responses in patients with RA and normal subjects as shown by Salvat et al. It may be
evident that MHC-derived peptides are not functional in the negative selection process in the thymus. However, in the same study the HLA DRB1*0401 allele was found to be tolerant in the same group of subjects, thus displaying a uniqueness of activity. The HLA DRB1*0401 subtype is that allele found in the extended haplotype B44-SC30-DR4.

The findings in this study, increased frequencies of the extended haplotype B44-SC30-DR4 and the C4B null allele in autistic subjects, are evidence for a possible association between autism and the major histocompatibility complex. Further studies may provide an actual relationship responsible for this association.
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