CHARACTERIZATION OF THE TNFa MICROSATELLITE'S RELIABILITY, MHC ASSOCIATIONS AND OCCURRENCE IN TWO ETHNICALLY DIFFERENT SLE POPULATIONS

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BY

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ABSTRACT

The major histocompatibility complex (MHC) encodes a diverse selection of genes, many of which have immunological functions. Several of these genes are polymorphic but are maintained in strong linkage disequilibrium as conserved extended haplotypes. Particular alleles at these loci or particular haplotypes have been found to be associated with increased susceptibility to various diseases. The TNFa microsatellite, with a variable number of dinucleotide repeats, is found 3.5 kb upstream of the tumour necrosis factor loci in the central region of the MHC. This project examined the reliability of the TNFa typing assay adapted for use in this laboratory. The TNFa microsatellite was then used to

a) further characterize extended haplotypes

b) investigate possible associations of alleles of TNFa with null alleles of the fourth component of complement (C4)

c) examine the possibility of disease susceptibility genes for systemic lupus erythematosus (SLE) in the TNFa region in two separate ethnic groups.

Fourteen TNFa alleles were identified and named in accordance with published literature. Repeat typings of samples and analysis of a large kindred were used to test

reliability of results obtained using TNFa microsatellite typing. A high level of consistency was demonstrated. The only errors in typing were in distinguishing heterozygosity with two consecutive alleles from homozygosity of the larger allele.

The presence of extended haplotypes, i.e. haplotypes sharing alleles at HLA-B,Bf,C4A,C4B and TNFa, was demonstrated. Although several extended haplotypes did exist, the most common extended haplotype in the population studied was HLAB8;TNFa2;BfS;C4AQ0;C4B1. It occurred in 15 of 75 unrelated chromosomes. There were examples of specific haplotypes sharing alleles at all loci except TNFa. There were five separate instances where this occurred in this data set. In the absence of HLA-DR data, the reasons for this are difficult to ascertain but possible explanations include multiple historic recombinations, rare extended haplotypes, and mutations of the original TNFa alleles occurring on the extended haplotypes.

The possible association of TNFa alleles with null alleles of C4 was investigated. If a strong association could be demonstrated, the TNFa allele could serve as a useful marker of the null alleles of C4 which are sometimes difficult to ascertain. Although TNFa2 occurred frequently with C4AQ0 on the extended haplotype HLA-B8;BfS;C4AQ0;C4B1 known to

contain a deleted C4A gene, no other clear association between particular TNFa alleles and C4 null alleles was observed. Similarly, in a population with null alleles identified by densitometry, there was no one TNFa variant occurring in all people with low C4A levels (relative to C4B). None of these "nulls" represented the 30 kb deletion commonly observed in Caucasians carrying the HLA-B8 extended haplotype.

The frequency of alleles of the TNFa microsatellite in Caucasian and Korean SLE patients was examined and compared to the appropriate ethnically matched controls. Both ethnic groups are known to have MHC associations with SLE, although not necessarily the same associations. We wished to use the TNFa microsatellite as a marker for the central region of the MHC to investigate whether there may be genes in the vicinity of the microsatellite, and in linkage disequilibrium with it, that may influence susceptibility to this disease. Our results demonstrated a significantly increased frequency of the TNFa2 allele in both ethnic groups. Although other genes exist in this region, the tumour necrosis factor loci, located 3.5 kb from this microsatellite, may be good candidate genes for susceptibility to SLE and are worthy of further study.

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ABBREVIATIONS

AH.....ancestral haplotype
APC.....antigen presenting cell
C2.....second component of complement
C4.....fourth component of complement
DNA.....deoxyribonucleic acid
HLA.....human leukocyte antigens
Ig G.....immunoglobulin G
LT.....lymphotoxin
LT-β.....lymphotoxin beta
MHC.....major histocompatibility complex
NZB.....New Zealand Black
NZW.....New Zealand White
PBL.....peripheral blood leukocytes
Q0.....quantity zero
RFLP.....restriction fragment length polymorphism
SLE.....systemic lupus erythematosus
TCRs.....T-cell receptors
TNF-α.....tumour necrosis factor alpha
TNF-β.....tumour necrosis factor beta
UTR.....untranslated region
bp.....base pair
Bf.....factor B
C3.....third component of complement
mRNA.....messenger ribonucleic acid
cDNA.....complementary DNA
PBMC.....peripheral blood mononuclear cells
dsDNA.....double-stranded DNA
ssDNA.....single-stranded DNA
EBV.....Epstein Barr Virus
rpm.....revolutions per minute
W.....watts
V.....volts
PCR.....polymerase chain reaction
PCR-SSP.....polymerase chain reaction-sequence specific primer

CHAPTER 1: INTRODUCTION

1.1 MHC Overview

The major histocompatibility complex (MHC) (discussed in Klein and Horejsi, 1997) in humans is located on the short arm of chromosome six in the band 6p21.3. It consists of at least four million base pairs (bp) of deoxyribonucleic acid (DNA) and contains more than 120 genes. It is subdivided into three *regions* referred to as Class I, II, and III. Although one third of the MHC has been sequenced, it is estimated that over 90% of the entire MHC region has no known function. The remaining 10% contains three *types* of genes. The MHC genes proper (or classical MHC) encode proteins with the ability to present peptides to the T-cell receptors (TCRs). These occur only in Class I and Class II regions. The second type of gene comprises those genes with immune function but which are not MHC genes proper. These include a diversity of genes found throughout the MHC including several genes in the Class III region which code for complement components. The third type of gene consist of those with no known immunological purpose. While occurring throughout the whole MHC, these genes are mainly concentrated in the Class III region and outnumber the other two types of genes found in the MHC. However, as

mentioned above, while the MHC Class III region does not contain any MHC genes proper, it does contain several immunologically important genes. In humans, the Class I and Class II molecules are often referred to as human leukocyte antigens (HLA) because of the site of their expression (reviewed in Klein and Horejsi, 1997; Kuby, 1997; Janeway and Travers, 1997).

The origins of the MHC genes are at least 450 million years old, having been found in all, except the oldest (the Agnatha), vertebrate classes. Although unproven, it seems likely that the three classes evolved independently of each other with the Class II the oldest (Klein and Horejsi, 1997).

1.1.1 Class I

Classical MHC Class I molecules found on almost all nucleated cells are involved in the presentation of peptides derived from intracellular parasites such as viruses to cytotoxic T cells. This initiates a cellular immune response that results in the destruction of the infected cells by the cytotoxic T cells. The three main MHC loci involved in this are the HLA-A, HLA-B, and HLA-C loci.

1.1.2 Class II

Classical MHC Class II molecules are found on specialized cells known as antigen presenting cells (APC). The APC

internalize protein from extracellular infectious agents, such as bacteria, by phagocytosis and present the foreign peptides bound to the Class II molecules on the APC's surface to helper T cells. This stimulates the humoral immune response and antibodies to the foreign proteins are produced. There are three main types of Class II molecules on the surface of APCs. These are HLA-DR, HLA-DQ, and HLA-DP. Their molecular biology is more complicated in that both chains of the surface molecule may have allelic forms and more than one gene may code for one of the chains. This, therefore, allows for great variety in the MHC Class II molecules.

1.1.3 Class III

Although the Class III region contains the greatest number of non-immunological genes, this region also includes many genes whose products are involved in the immune system such as the genes for tumour necrosis factor alpha (TNF-α), tumour necrosis factor beta (TNF-β) and various components of complement. The four components of complement encoded in the central region of the MHC are the second component of complement (C2),factor B (Bf), and two isotypes of the fourth component of complement (C4A and C4B). These four complement genes are tightly linked and no crossovers have been observed between these loci in several hundred meioses (Alper et al., 1983). In 1981, Awdeh and colleagues used the term complotype

to refer to the haplotype of these four closely linked complement genes that always appear to be inherited as a single unit.

1.1.4 Polymorphism and Linkage Disequilibrium

MHC loci, especially the MHC genes proper, are highly polymorphic with a large number of alleles at each locus, most occurring at appreciable frequencies. New alleles are being discovered as more populations are being HLA-typed. Variation is mostly concentrated in the region of peptide binding. Because of this, different MHC molecules may differ in their ability to interact with a particular antigenic peptide. This may offer one MHC type a greater advantage over another in presenting given peptides of infectious organisms to stimulate an immune response. Although polymorphism does exist in the Class III MHC loci, these genes are considerably less polymorphic than the classical MHC loci.

Although highly polymorphic, many of the MHC genes are tightly linked and inherited as a haplotype. Alleles of the MHC genes are often said to be in linkage disequilibrium; that is, particular alleles of two or more genes are found together more often than would be predicted by their individual frequencies in the population (Bodmer and Bodmer, 1978). There are several combinations of alleles at multiple loci that show significant linkage disequilibrium. Alper and

colleagues (1989) referred to these as extended haplotypes and demonstrated that the frequency of twelve of these haplotypes, when combined, accounted for approximately 30% of all MHC haplotypes in their Caucasian sample. Several groups have shown that examples of the same extended haplotype from unrelated individuals have the same overall genomic organization and DNA composition at all sequences in the MHC between HLA-B and HLA-DR so far studied (Zhang et al., 1990; Pei et al., 1991; Abraham et al., 1992; Wu et al, 1992; Degli-Esposti et al., 1992a). Because of this evidence, many of these researchers refer to these haplotypes as ancestral haplotypes (AH). They believe that the ancestral haplotypes are identical because they have been conserved by descent from a very remote ancestor (Degli-Esposti et al., 1992a). In their paper, Degli-Esposti and coworkers (1992a) observed 22 ancestral haplotypes that accounted for 38% of unrelated Caucasian haplotypes studied. These were full HLA-B--HLA-DR ancestral haplotypes, but another 35% contained fragments of the ancestral haplotypes presumably generated by ancient crossover between two ancestral haplotypes. The studies of Degli-Esposti and coworkers (1992a) and Alper and colleagues (1989) revealed that ancestral or extended haplotypes occurred more frequently than their putative recombinants.

Certain MHC alleles are known to occur more often in patients with particular diseases (especially autoimmune

diseases) than in healthy individuals. These loci, or nearby loci in linkage disequilibrium, are believed to be susceptibility loci such that having a particular allele at that locus increases the risk of developing that disease. However, the disease associated allele is not found in all patients and may also be found in healthy individuals, reflecting a complex, multifactorial etiology for that disease. One of the strongest associations has been found with HLA-B27 and ankylosing spondylitis, an autoimmune disease of the vertebral joints. Approximately 90% of patients carry this allele while it is present in only 9% of healthy individuals (Klein and Horejsi, 1997). Similarly, the DQB1*0602 allele is significantly increased in individuals with narcolepsy across all ethnic groups (Mignot et al., 1997). The association is strongest with those patients exhibiting cataplexy, a symptom consisting of muscle weakness brought on by emotion. In the study by Mignot and colleagues (1997), the frequency of the DQB1*0602 allele in patients with severe cataplexy was 94.8% whereas it was only 24% in controls. Although most associations of disease with alleles at particular loci are considerably less definite than these examples, particular alleles or haplotypes often represent an increased risk of developing certain diseases. The genes involved in this increased risk of developing a particular disease are called disease susceptibility genes.

1.2 Fourth Component of Complement

The Class III region of the MHC contains a diverse selection of immunologically relevant genes including those for the complement components and the tumour necrosis factor loci. C2 and the C4 loci encode complement components which are involved in the classical complement pathway while factor B, the product of the Bf gene, is involved in the alternative complement pathway. Although the means of activation of these two pathways differ--the first requires an activating substance such as antibody, while the latter occurs continuously at a low level--both pathways converge on the lytic pathway. Both pathways converge by generating a third component of complement (C3) convertase. However, the C3 convertases are generated by different routes and are structurally different. A fragment of the C4 or Bf molecule forms part of the C3 convertase in the classical or alternative complement pathways respectively. Both the C3 convertases have the same activity and thus the later events involving lysis and death of the target cell follow the same route (Janeway and Travers, 1997). The C4 molecule in the classical complement pathway is also involved in clearing of antibody:antigen complexes present in the circulation (Schifferli et al., 1985).

1.2.1 Isotypes of C4: C4A and C4B

There are two isotypes of the fourth component of complement: C4A and C4B, with several alleles at each of the tandem loci. This makes them the most polymorphic loci of the central region of the MHC. Not only are they polymorphic, but duplication(s) of loci have been demonstrated. Furthermore, null alleles, defined as the absence of the protein product and designated Q0 (quantity zero), have been demonstrated to occur at appreciable frequencies for both loci. Null alleles at the phenotypic level have traditionally been identified using densitometry and comparison of the staining of C4A to C4B proteins. This assumes a gene dose effect, i.e. equivalent amounts of C4A and C4B proteins are produced if there are the same number of genes of C4A and C4B. However, Hammond and colleagues (1992) did show that there was considerable phenotypic overlap between genotypic groups with different numbers of C4 genes. Family studies can be very useful in identifying true null alleles. At the DNA level, it has been shown that about half of the C4 genes typed as null are in fact deleted (Schneider et al., 1986; Carroll and Alper, 1987) and the C4A null in particular is deleted most often as part of a 30 kb deletion encompassing the C4A locus but not the C4B locus (Uring-Lambert et al., 1987). This deletion frequently occurs as part of the extended ancestral haplotype 8.1: A1;Cw7;B8;C2C;BfS;C4AQ0;C4B1;DR3. In the case

of non-deleted null alleles, Partanen and Campbell (1989) and Braun and colleagues (1990) reported evidence suggesting that nondeleted null alleles at C4A were probably caused by inactivating point mutations or small insertions or deletions. However, most non-deleted C4B null alleles probably represent gene conversion to C4A-like genes which may be expressed and typed as C4A proteins.

The two isotypes of C4 differ by only 14 nucleotides, but they do show different efficiencies in some of their functions. For example, haemolysis was 3.2 fold more efficient by C4B than by C4A, but C4A was 1.7 fold more efficient at inhibiting immune precipitation of antigen:antibody complexes than C4B (Schifferli et al., 1986; Paul et al., 1988; Kishore et al., 1988). This may suggest that C4A is more important in the immune clearance reaction.

1.3 Tumour Necrosis Factor

The tumour necrosis factor loci are contained within the central region of the MHC. In 1985, messenger ribonucleic acid (mRNA) for tumour necrosis factor alpha was isolated and the complementary DNA (cDNA) synthesized (Marmenout et al., 1985). The mature protein sequence is 157 amino acids long and about 80% homologous to mouse TNF. In the same year, the gene encoding TNF was located in a human genomic library

(Shirai et al., 1985). The similarities and differences between tumour necrosis factor alpha (TNF-α) and lymphotoxin (LT), later also known as tumour necrosis factor beta (TNF-β), were examined by Nedwin and colleagues (1985). The genes encode proteins of similar structure and activity but with different expression in particular cells. They share approximately 30% amino acid homology. Each gene contains three intervening sequences with the last exon sharing the greatest homology and coding for more than 80% of the mature protein for both TNF-α (157 amino acids) and TNF-β (171 amino acids). During the following year Nedospasov and colleagues (1986) determined that the TNF-α and TNF-β genes are tandemly arranged and in close linkage. The predicted amino acid sequences for murine and human lymphotoxin (LT) indicated that the proteins are about 72% homologous (Gardner et al., 1987). Another structurally similar protein, also found to be encoded by a gene in the MHC, was named lymphotoxin-β (LT-β). It forms a heteromeric complex with lymphotoxin on the surface of activated T cells, B cells, and lymphokine-activated killer cells (Browning et al., 1993).

Although produced by different sources, TNF-α and TNF-β (mainly produced by macrophages and activated T lymphoctyes respectively) have very similar biological effects, partly reflecting their homology and also the fact that both act via the same receptor. One of the main biological activities of

both is endothelial cell activation, but TNF-α is also significantly involved in local inflammation while TNF-β is involved with killing of target cells. Both of these cytokines have a multitude of diverse functions and are an integral part in the regulation of the immune system (Klein and Horejsi, 1997; Paul and Ruddle, 1988).

1.3.1 Polymorphism of the TNF Loci

There does not seem to be significant allelic variation in the protein structure of either TNF-α or TNF-β. The only amino acid difference noted to date has been at position 26 of TNF-β, with an asparagine replacing a threonine (Messer et al., 1991; Voigt et al., 1992). Other studies of the TNF loci indicated only a low degree of DNA polymorphism. A restriction fragment length polymorphism (RFLP) in the 3'-untranslated region (UTR) of TNF-β was demonstrated using EcoRI restriction endonuclease with a frequency of about 6% for the less common allele (Partanen and Koskimies, 1988). After extensive testing with restriction endonucleases, another RFLP in the first intron of the TNF-β gene was demonstrated with the NcoI enzyme (Fugger et al., 1989a). The TNFB*2 (lack of restriction site) was present in 64 to 72% of haplotypes (Fugger et al., 1989a; Webb and Chaplin, 1990). However, the less common TNFB*1 allele was found to be strongly associated with the HLA-A1;HLA-B8;HLA-DR3 haplotype

(Fugger et al., 1989a; Badenhoop et al., 1990; Pociot et al., 1991; Bettinotti et al., 1993). Other polymorphisms have been identified in the tumour necrosis loci including a polymorphism within the 5' regulatory region of the TNF-α gene at position -308 in which a guanine is substituted by an adenosine in the uncommon (TNF2) allele (Wilson et al., 1994). This also was found to be strongly associated with the B8;DR3 haplotype in the Northern European population (Wilson et al., 1994) and with DR3 in the Chinese population (Chen et al., 1997).

1.3.2 Disease Associations

Although protein variation for TNF-α and TNF-β is rare, there have been reports of altered levels of secretion in some diseases such as insulin-dependent diabetes mellitus (IDDM) (Sachs et al., 1990; Pociot et al., 1991). The possibility exists that there could be regulatory variants of these genes. Considering this, and their location in the MHC and their function, these genes make promising susceptibility genes.

Several authors have looked at the NcoI polymorphism as a marker for the TNF loci and the central region of the MHC in several conditions. Some, such as early primary allograft loss (Mahoney et al., 1996) and rheumatoid arthritis (Campbell et al., 1994), showed no significant differences between normals and controls. However, the same polymorphism did show a

decreased frequency of the 10.5 kb allele (TNFB*2) in primary biliary cirrhosis (Fugger et al., 1989a) and, in Graves disease, there were fewer homozygotes for this allele and more heterozygotes compared to controls (Badenhoop et al., 1992). Also, in patients with IDDM, the TNFB*2 allele was carried on the DR3 haplotype three times more frequently than in controls, suggesting that this combination may contribute to susceptibility in IDDM (Pociot et al., 1991). The TNF polymorphism at position -308 in the TNF-α gene has also been examined in different patient groups. No significant differences in allele frequencies between patient groups and controls have been found in ankylosing spondylitis (Verjans et al., 1994), Felty's syndrome (Brinkman et al., 1994), and IDDM (Pociot et al., 1993a).

1.3.3 Levels of Secretion

There is evidence to suggest a genetic basis for variation in the levels of secretion of TNF, although there are contradictory reports on this matter. Jacob and colleagues in 1990 examined individual differences in the production of TNF-α. By subjecting unseparated peripheral blood leukocytes (PBLs) to a variety of different inducing agents and concentrations, they demonstrated significant interindividual differences in the level of TNF inducibility The interindividual differences between high and low TNF-α

producers were evident in all methods of activation and at different dosages of inducers (Jacob et al., 1990).

Although in the study by Jacob and colleagues (1990) cells from healthy males and females had similar levels of inducible tumour necrosis factor, individual male levels were stable when randomly tested 3-5 times at later intervals while there was considerable fluctuation in the female levels. This fluctuation was greatly reduced in the postmenopausal women and would seem to indicate partial regulation of TNF-α by sex hormones.

1.3.3A Correlation of Secretion with HLA and RFLPs

Jacob and colleagues (1990) also investigated associations between MHC Class II and TNF-α inducibility. They found that DR2 and DQw1 subjects were low producers and DR3 and DR4 subjects were high producers. A heterozygote DR2,3 or DR2,4 was a high producer indicating a dominant effect exhibited by the DR3 or DR4 haplotypes. No correlation was found between MHC Class I genotype and TNF-α production even though Class I genes are closer than Class II to the TNF-α locus. No correlation between TNF-β production *in vitro* and MHC class I or class II genotype could be demonstrated nor could they show any differences in TNF-α or TNF-β inducibility with the NcoI RFLP. However, another study (Pociot et al., 1991), did show significantly lower TNF-α inducibility from

individuals who were homozygous for the TNFB*2 allele.

Several other investigators have also investigated the relationship between levels of *in vitro* TNF production and MHC polymorphisms. Sachs and colleagues (1990) found that there was no correlation between HLA phenotype and the amount of TNF-α or TNF-β secreted from stimulated peripheral blood mononuclear cells (PBMC). On the other hand, Abraham and coworkers (1993a) found that ancestral haplotypes did correlate with the activity of TNF-α from unstimulated cells. There appeared to be a trend of increased production of TNF-α from the 8.1 ancestral haplotype (which also includes the TNFB*1 NcoI polymorphism). The relationship of high and low secretors to certain alleles and extended haplotypes was also demonstrated by Pociot and colleagues (1993b) but only for TNF-α. No correlation could be found with TNF-β.

1.4 Systemic Lupus Erythematosus

1.4.1 Disease Characteristics

Systemic lupus erythematosus (SLE) is an autoimmune disease involving multiple organ systems. It has diverse clinical manifestations with skin rash, joint pain and spontaneous remission on one end of the spectrum and severe and progressive kidney involvement (lupus nephritis) at the

other end. For a diagnosis of SLE, at least four of eleven criteria need to be present as defined by the American Rheumatism Association (Tan et al., 1982). Because of the multitude of different phenotypes involved, it is unclear whether SLE may be considered one syndrome with variable expression or whether it is better described as a group of related conditions (Kotzin, 1996).

Patients have immune abnormalities indicative of B cell hyperactivity: hypergammaglobulinemia, increased amounts of antibodies reactive with self determinants (including nuclear antigens), and increased numbers of circulating antibody-producing cells (Steinberg and Klinman, 1988). The most common feature of SLE patients is immunoglobulin G (Ig G) autoantibody production with elevated serum levels of antibodies to nuclear components. It is believed that this is the major pathogenic mediator in SLE, but the mechanisms by which this occurs are variable and not always well elucidated (Vyse and Kotzin, 1998).

The overall prevalence of SLE is approximately 1 in 2000 in the United States with women of childbearing age primarily affected. The female to male ratio during these years is >8:1 (Kotzin, 1996) and the chance of a Caucasian female developing SLE in her lifetime is approximately 1 in 700 with an incidence two to four times greater for Blacks and Hispanics. According to Hopkinson and colleagues (1994), the overall one

year prevalence rate in Great Britain was 24.7 per 100,000 persons, with the highest rate in Afro-Caribbeans (207/100,000), then Asians (48.8/100,000) followed by whites (20.3/100,000).

1.4.2 Etiology of SLE

Etiology of SLE is unknown but there is considerable evidence that the development of SLE involves both genetic and environmental factors. First, there is familial clustering of the disease with a lambda s value (risk ratio of affected sib pair to population prevalence) estimated to be between 10 and 20 compared to a value of 1 if there is no evidence of familial clustering or genetic factors (Vyse and Kotzin, 1998). There is also a concordance rate of 24% for monozygotic twins. However, it is apparent from the lack of complete concordance in twin studies that there are other non-genetic factors involved. This is supported by Hess and Farhey in their 1994 review, and by Lewkonia (1992) in her discussion about the different prevalence rates of SLE between Chinese groups living in separate parts of the world, indicating that other factors besides genetic makeup are important in the pathogenesis of this disease.

Nevertheless, the significance of genetic involvement in this disease can not be denied. Reveille and coworkers (1998) looked at the effects of various genetic markers,

socioeconomic factors and ethnic factors in the onset and clinical manifestations of SLE. They found that immunogenetic and ethnic factors appear to be more important than socioeconomic factors in influencing disease phenotype. For example, cardiac involvement was more frequently seen in the Hispanic population than in the Caucasian population.

1.4.2A MHC Associations with SLE

Several MHC associations with SLE have been demonstrated in a variety of populations. Associations with several DRB1 alleles have been demonstrated including DRB1*0301 in the Caucasians, DRB1*1503 in African-Americans, DRB1*08 in Hispanics, (Reveille et al., 1998) and DRB1*1501 in Japanese (Tomita et al., 1993). Besides these associations, one of the most pervasive associations has been with the null allele of the fourth component of complement. Significant increases in the frequency of the null allele of the fourth component of complement, especially of the A isotype, have been demonstrated in Caucasian (Kemp et al., 1987; Partanen et al., 1988; Goldstein et al., 1988; Fronek et al., 1988), Japanese (Yamada et al., 1990) and Korean SLE (Hong et al. 1994). Importantly, in the last two groups it has not been associated with the B8;DR3 haplotype, increasing the likelihood that it is the C4 locus that is the susceptibility locus on this extended haplotype.

1.4.3 New Zealand Hybrid Mouse Model of SLE

Animal models can be very useful in helping to understand factors that contribute to a disease. Several murine models of spontaneous lupus-like disease exist. These include the BXSB and the MRL mice models but the most widely studied is the New Zealand hybrid model. In this model, a New Zealand Black (NZB) mated with a New Zealand White (NZW) produce offspring [(NZB X NZW)F1] that develop a disease similar to lupus nephritis and produce high levels of autoantibodies to nuclear antigens including double-stranded DNA (dsDNA) (Vyse and Kotzin, 1998). In this cross, 95% of the female F1 animals die from renal disease by 12 months of age. However, less than 5% of the parental mice develop severe glomerulonephritis during the first year of life and rarely produce high levels of antibodies to nuclear proteins (Babcock et al., 1989). NZB mice do show signs of autoimmunity but not the range or severity of the offspring. The NZW parent does not show signs of autoimmunity.

1.4.3A Genetic Contribution of NZB and NZW Mice

Raveche and colleagues (1981) investigated the genetic nature of autoimmunity in the New Zealand mice and determined that multiple genes (at least six) were responsible for the eight autoimmune traits characteristic of NZB mice. Also, production of anti-T cell antibodies and antibodies to single-

stranded DNA (ssDNA) were each found to be inherited as independent traits controlled by unlinked genes. Other non-MHC genes have been implicated in other mouse models as well (Garchon and Bach, 1991; Garchon, 1992; Cohen and Eisenberg, 1991).

Babcock et al. (1989) determined that it was one or more genes in or closely linked to the mouse MHC that was the major NZW contribution in the (NZB X NZW)F1 disease. The probability of developing renal disease in the hybrid is not absolute (approximately 90%) while having only the NZB MHC haplotype still confers a small (approximately 10%) risk of developing severe renal disease.

1.4.3B The Role of TNF-α

What is it specifically about the NZW mouse MHC that confers the increased risk of developing severe renal disease in the context of the NZB genetic background? One theory suggests that the predisposing element is the tumour necrosis factor gene in these animals. There are several lines of evidence to support this hypothesis. The NZW parental strain produces very low levels of TNF-α in peritoneal exudate cells (Jacob et al., 1991a). Mice in the F1 generation have TNF levels closer to that of the NZW than to the NZB parent, and therapy with recombinant TNF-α results in a decrease in the development of lupus nephritis in the F1 mice. Two other

strains of mice that develop lupus-like disease, the MRL and BXSB mice, produce moderate and high levels of TNF respectively. Interestingly, although some effect by TNF therapy on suppressing disease in MRL mice could also be shown, none could be shown in BXSB mice. The protective effect seems to be evident only when there are low levels of endogenous TNF.

Several groups (Jongeneel et al., 1990; Jacob et al., 1991b; Jacob and Hwang, 1992) were interested to see if there was a genetic explanation in the MHC for this effect. They investigated RFLPs in various autoimmune and non-autoimmune strains of mice but did not find a correlation with autoimmunity or production of TNF. In the same manner they investigated a polymorphic (AC)n repeat in the promoter region of the TNF-α gene. It was established that strains carrying the same MHC haplotype also carried the same (AC)n repeat allele, although the alleles may be found in other MHC haplotypes as well. Although all the autoimmune mouse strains did not show that same allele, the NZW mice had a unique microsatellite allele of 17 AC copies. It was proposed that this allele may be associated with a regulatory or structural defect.

In an effort to clarify whether the difference in level of expression of TNF genes in various strains of mice was the result of differential transcription or perhaps differential

translation or induction, Bazzoni and Beutler in 1995 examined the levels of mRNA in different strains of mice. Cells from NZW mice contained less mRNA than did cells from NZB mice when unstimulated or following activation with two different LPS concentrations. Sequence differences in the 3'-UTR of the TNF-α gene allowed the investigators to distinguish which allele was contributed by each parent in the (NZB X NZW)F1 mice. In these mice there was approximately equal transcription of mRNA from each allele. Therefore, the lower concentration of TNF mRNA in the NZW strain is extrinsic to the TNF gene itself.

In 1996, Jacob and coworkers revealed a regulatory role for the 3'-UTR in the genetic predisposition to lupus-like autoimmune disease. The variability of TNF-α production in various mouse strains is MHC associated. Mice with the same MHC produced similar levels of cytokine. A cooperation between the upstream regulatory region and the 3'-UTR has been demonstrated and mutational analysis of various mouse strains in the 3'-UTR demonstrated mutations which were similar in strains showing a particular production pattern. Analysis of TNF gene regulation using a functional reporter gene assay indicated that the insertional mutations in the NZW 3'-UTR down-regulates the amount of product produced. However, the level of mRNA was not affected. Therefore, in agreement with Bazzoni and Beutler (1995), these investigators concluded that

there is no necessary correlation between RNA transcript level and TNF protein production.

1.4.4 TNF-α in Human SLE

There is considerable evidence from the New Zealand hybrid mouse model to suggest a role for TNF-α in some types of murine SLE. This makes TNF a promising candidate locus for susceptibility to SLE in the human condition as well. Variation in the amino acid composition of TNF-α and TNF-β is rare. However, altered levels of secretion of these proteins have been reported in some diseases and have demonstrated associations with some MHC genes (section 1.3.3A). The levels of secretion associated with the MHC is of particular interest because many diseases, such as SLE, have also demonstrated MHC associations. It is possible that these MHC genes may be markers in linkage disequilibrium with the primary disease susceptibility locus. Of the two HLA-DR haplotypes (DR2 and DR3) associated with SLE, only DR2, DQw1 positive SLE patients show an increased risk of developing nephritis. These DR2, DQw1 positive patients produce low levels of TNF-α (Jacob et al., 1990). DR4 is negatively associated with nephritis. As with healthy individuals, only DR2, DQw1 SLE patients show a significant association with low TNF production. In fact, DR3 and/or DR4 positive SLE patients are negatively associated with low TNF production, just as with normal DR3 and DR4

subjects. Thus, development of nephritis does seem to correlate with low TNF production (Jacob et al., 1990).

In the hopes that the previously mentioned polymorphisms identified in the tumour necrosis factor loci (section 1.3.1) might be a better marker for regulatory allelic variation of these loci, several investigators have examined them in a variety of SLE populations. Fugger and coworkers (1989b) and Bettinotti and colleagues (1993) both found an increase of the TNFB*1 phenotype in SLE but only in the presence of the B8;DR3 haplotype which is known to be in linkage disequilibrium with this allele. In a small group of 20 Japanese patients with SLE, Tomita and coworkers (1993) also found an increased frequency of this allele but not in association with the B8;DR3 haplotype (which is rare in both normal individuals and SLE patients in the Japanese population). However, whether it is in linkage disequilibrium with another haplotype was not investigated.

Similarly, although the TNF2 allele was significantly increased in the Caucasian SLE population examined by Wilson and colleagues (1994) and Danis and colleagues (1994), so was B8 and DR3 and no independent association with any of the alleles could be demonstrated. Another study examined the TNF2 allele in Caucasian and in black South African SLE patients and found an increased frequency of the TNF2 allele in only the Caucasian SLE which was strongly associated with

the DR3 haplotype (Rudwaleit et al., 1996). A marginally significant increase of the TNF2 allele (p=0.049) was demonstrated in 105 unrelated Chinese patients in Taiwan by Lu and coworkers (1997). However, in their 100 Chinese patients, Chen and colleagues (1997) could find no difference in the frequency of the TNF2 allele between the control and SLE populations.

1.5 TNFa Microsatellite

Because the previously mentioned RFLPs are only biallelic, the informativeness of these studies is limited. A more polymorphic marker would have a better chance of demonstrating an association if one did indeed exist. Weber and May in 1989 identified a group of highly polymorphic markers found throughout the genome which are referred to as microsatellites or simple sequence repeats (Litt and Luty, 1989). These are tandemly repeated short DNA segments with a variable number of repeats. One of the most common is the dinucleotide repeat in which two base pairs are tandemly repeated. Several of these have been identified within the center portion of the MHC (Nedospasov et al., 1991) and one in particular, a CA repeat named TNFa located approximately 3.5 kb upstream of the TNF loci, proved to be very polymorphic with 12 alleles identified (Jongeneel et al., 1991). This

polymorphic locus may be a useful marker for the central region of the MHC, including the TNF loci (Figure 1.1.). It may further help to elucidate the contribution of this region to a variety of MHC associated diseases, including SLE.

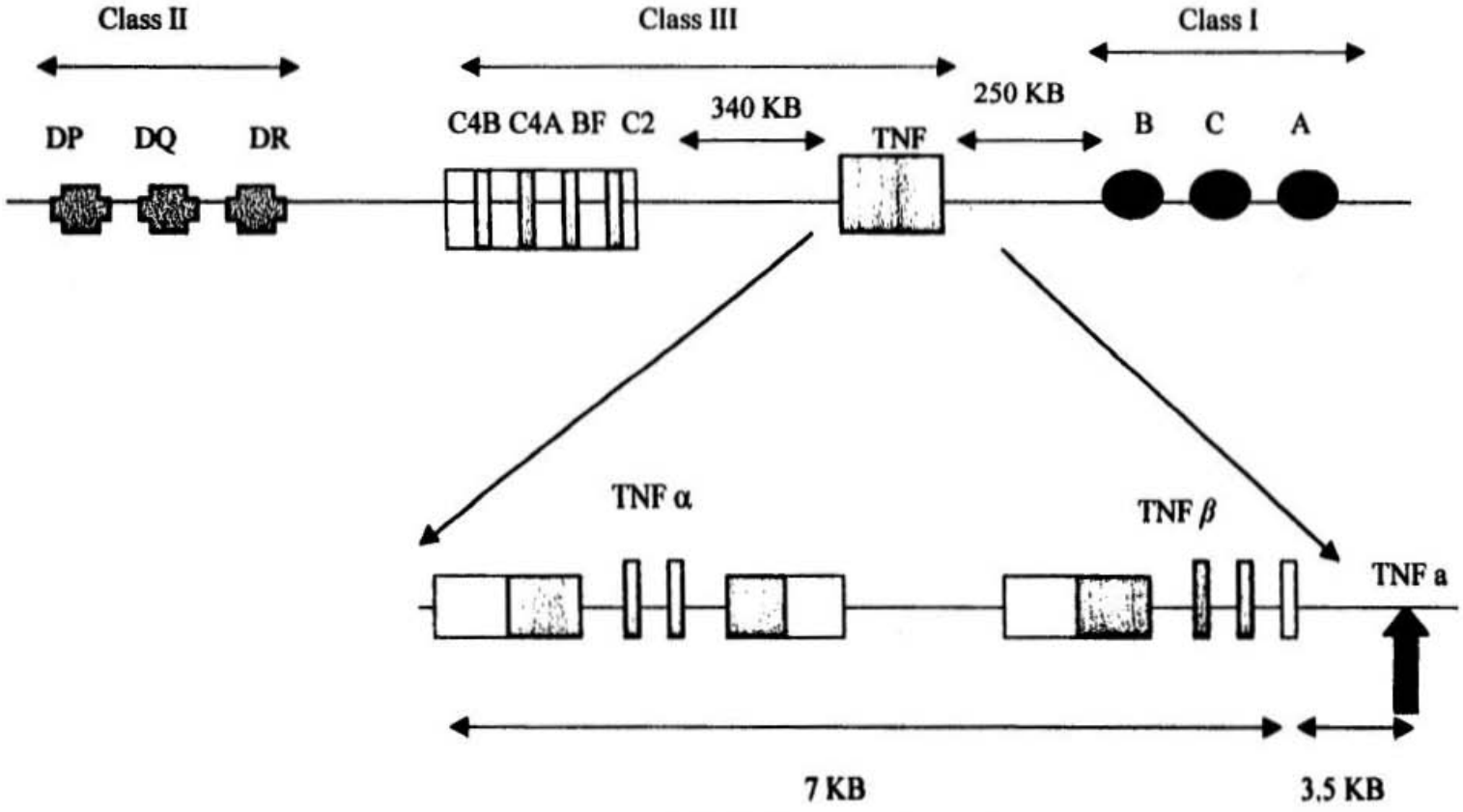

Figure 1.1: The human major histocompatibility complex, including the loci for TNF genes and the microsatellite TNFa. The upper panel shows the arrangement of loci for Class I, II and III genes. Class III genes include those for complement components C2, factor B, C4A and C4B as well as TNF. Class I and Class II MHC genes include those that code for HLA-A,-B,-C,-DR,-DQ and -DP. The lower panel shows details of the arrangement of the genes for TNF-α and TNF-β. Open boxes are non-translated regions. The location of the microsatellite TNFa is indicated by the open arrow.

CHAPTER 2: AIMS AND OBJECTIVES

This project was carried out in a laboratory involved in the study of MHC variation and MHC disease associations. The MHC contains a diverse selection of genes with immunological function. Many of these genes are polymorphic but are often maintained in strong linkage disequilibrium as conserved extended haplotypes. Many investigators believe that examples of these extended or ancestral haplotypes in apparently unrelated individuals represent haplotypes that have descended from a very remote ancestor and have been conserved *en bloc* over many generations. Particular alleles at some MHC loci or particular extended haplotypes have been found to be associated with increased susceptibility to various diseases. Because of the extended haplotypes, it can be difficult to identify which of the many genes in linkage disequilibrium is primarily responsible for susceptibility to a particular disease. The TNFa microsatellite, found in the Class III region of the MHC as described by Jongeneel and colleagues (1991), would provide a DNA based technique for MHC typing to be used in this laboratory to augment the serological typing of many Class I, Class II, and Class III MHC components already established in this laboratory.

As part of its work in the MHC, this laboratory was

interested in the typing of C4 protein products encoded for by genes (C4A and C4B) found in the Class III region of the MHC. C4 typing can be complicated because of duplication(s) of loci and null alleles on haplotypes. Null alleles, in which no protein product is produced by that allele, have been implicated in susceptibility to certain autoimmune diseases but are difficult to ascertain in the absence of informative family data. Densitometry is often used to identify null alleles in subjects based on relative staining intensities of C4A to C4B bands after electrophoretic separation of bands on gels and staining of products. However, the use of densitometry can be subject to miscalculation of the number of nulls in a sample. Nevertheless, if a strong association between null alleles of C4 and particular variants of TNFa could be found, this would complement uninformative family studies and densitometry to allow greater confidence in assigning null alleles to individuals.

Similarly, the TNFa microsatellite would provide another marker for the central region of the MHC which is implicated in several diseases such as SLE. Association of SLE with various MHC genes has been documented for many different ethnic groups. Often these associations differ in the different ethnic groups, possibly reflecting separate etiologies in this complex, multifactorial disease. However,

the different associations may also indicate that these genes are not the primary susceptibility loci, but may, in fact, be in linkage disequilibrium with another locus that is important in the etiology of this disease. Thus, it would be expected that the closer the marker locus is to the susceptibility gene, the greater the association between the disease and the marker locus (in linkage disequilibrium with the susceptibility locus). Therefore, if a gene close to the TNFa microsatellite is important in susceptibility to SLE, altered allele frequencies between normal and disease groups for the TNFa microsatellite could be expected, and the association of SLE with this marker might be stronger than for other genes in the MHC region.

Based on this rationale, the specific objectives for this thesis were:

1. develop the assay for typing of the TNFa microsatellite and test the accuracy and reliability of this method.
2. to describe the TNFa alleles as part of extended MHC haplotypes.
3. to analyze whether any TNFa alleles are associated with null alleles of C4. In the first part of this objective, definite nulls, determined from informative haplotypes from an extended family, will be examined for association with particular TNFa microsatellites. Secondly, a population of

null alleles of C4 assigned using densitometry will be examined to determine if a specific TNFa variant is associated with null alleles of C4 assigned by this method.

4. to determine if a particular TNFa variant is increased in SLE patients from two different ethnic groups (Newfoundland Caucasian and Korean) compared to ethnically matched controls.

CHAPTER 3: MATERIALS AND METHODS

3.1 Subjects

Material to be used for DNA extraction for this study came from a variety of different sources. The initial phase was a family study using approximately 300 stored samples of known MHC haplotypes collected and described in 1976 as part of a study of a large kindred in Western Newfoundland. This was followed by the collection of two unrelated, normal and SLE populations, one set Caucasian and the other Korean.

3.1.1 Family Material

The family material (WC series) came from a large extended family living on the northwest coast of Newfoundland. The three communities from which the family material originated had been subjected to intensive study by the Immunology Research Laboratories (Faculty of Medicine, Memorial University of Newfoundland) during the West Coast Health Surveys of 1974 and 1976 and samples of three generations were available (Marshall, 1975). Because of this, serological information existed on HLA-A, HLA-B, and later study of serum samples contributed information on factor B and

on C4A and C4B. Extensive pedigree analysis had been carried out and haplotypes of these MHC loci had been derived for many people. This served as an excellent source of family material for haplotype analysis of the microsatellite and identification of microsatellite-HLA-complement haplotypes. Peripheral blood leukocytes (PBLs) from some individuals had been stored at -70 °C during the course of the 1974, 1976 and the follow-up 1979 visits at approximately 5,000,000 cells/mL.

Factor B and C4 typing were done according to the technology at the time. Bf typing was done in accordance with the method described by Alper and coworkers (1969) using the technique of immunofixation electrophoresis. Samples were assigned alleles S, F, F1, or S1. Alleles of C4A and C4B were identified using the method of Awdeh and Alper (1980) by immunofixation electrophoresis of desialated plasma samples. Five C4A alleles (A6, A4, A3, A2, and AQ0) and five C4B alleles (B4, B3, B2, B1, and BQ0) were identified in this material. On occasion, alleles were scored as A3* or B1* where, based on family data available, it was possible to assign to a haplotype either the common allele, A3 for the A locus or B1 for the B locus, or the null A or B allele. In

other words, the family material available was not fully informative and A3* means A3 or AQ0 and B1* means B1 or BQ0.

3.1.2 Normal Populations

(i). Eighty-four males and twenty-five females between the ages of twenty and thirty years of age , many from the Faculty of Medicine and other hospital and lab personnel, made up the Newfoundland population sample. All were healthy individuals, not on any medication. Blood samples were collected by venipuncture in Vacutainer tubes between 8:00am and 12:00 midday with samples from no more than five individuals being collected at any one time. Generally, 4-5 ACD tubes and one clot tube were collected. As these samples were being used for another study as well, only the polymorphonuclear cells were available for DNA extraction. Blood samples were prepared by Ficoll-Hypaque (Sigma, Oakville,ON) centrifugation by Laura Sampson-Murphy (Immunology Research Laboratory) and the polymorphonuclear layer removed by pipetting the top of the lower blood layer, where the polymorphonuclear cells reside, and putting it into 15 mL centrifuge tubes. The tubes were then filled with Hanks solution (Gibco/BRL, Burlington, ON) or RPMI (Gibco/BRL) and stored at 4 °C for up to five days prior to DNA extraction.

(ii). DNA samples from 52 Korean people were provided at a concentration of 1μg/μL as part of a collaboration between this lab and Ms. Gi Hong at the Women's University in Korea. Information on several Class I, Class II and Class III MHC loci typed in their lab was also included.

3.1.3 Disease Populations

(i). Samples from twenty patients with systemic lupus erythematosus (SLE) were previously collected during the summer of 1991 and PBLs or Epstein Barr Virus (EBV) transformed B cells were frozen and stored in liquid nitrogen. Samples from seven other SLE patients were also collected at the time of this study, and fresh blood was extracted by venipuncture into EDTA Vacutainer tubes from which DNA was extracted. This gave a total of twenty-seven Caucasian SLE patients.

(ii). DNA from forty-eight Korean SLE patients was provided by Ms. Gi Hong in Korea. The concentration of the samples was 1μg/μL. MHC data on some Class II and Class III loci were also provided as well as information on C4 restriction digests (Hong et al., 1994).

3.1.4 Workshop Cell Lines

The Tenth International Histocompatibility Workshop cell lines (Yang et al., 1989) were a gift from Dr. William Marshall's lab (Faculty of Medicine, Memorial University of Newfoundland).

3.2 DNA Extractions

DNA was extracted according to the methods in use in the laboratories of Dr. R. Green and Dr. B. Younghusband (Faculty of Medicine, Memorial University of Newfoundland, personal communication). Some modifications were made as indicated below.

3.2.1 Whole Blood

Blood was collected in 5 or 7 mL EDTA Vacutainer tubes. Tubes were stored at room temperature for up to three days from the time of collection. Five volumes of warm (37 °C) ammonium chloride (Sigma):tris solution (Sigma) (0.14 M NH_4Cl; 0.017 M Tris-HCl, pH 7.65) were added to one volume of blood (5-7 mL) in a 50 mL centrifuge tube. This was incubated at 37 °C for five minutes and followed by centrifugation at 2500 revolutions per minute (rpm) for five minutes. The lysed red blood cell mixture was decanted, the white cell pellet

resuspended in 10 mL of 0.85% sodium chloride solution, centrifuged at 2500 rpm for 5 minutes and the supernatant poured off.

Once the white cell pellet was obtained, the 'salting out' procedure by Miller et al. (1988) for the extraction of DNA from buffy coats was followed with minor alterations. The pellet was resuspended in 3 mL of nucleic lysis buffer (10.0mM Tris-HCl, 400.0mM NaCl [BDH, Toronto, ON] and 2.0 mM Na_2EDTA [Sigma], pH 8.2), transferred to a 15 mL centrifuge tube and 0.2 mL of 10% SDS (BioRad, Richmond, ON) and 0.5 mL of pronase E (Sigma) solution (3mg/mL in 1% SDS, 2mM EDTA) added. The cell lysate was left to digest overnight at 37°C. Alternatively, digestion could be completed in 2h at 55 °C or left for three nights at room temperature. Leaving samples at 37 °C for two nights did not seem to have a detrimental effect on the extraction.

After digestion was complete, 1 mL of saturated sodium chloride (approximately 6M) was added to the tube and shaken vigorously for fifteen seconds, followed by centrifugation at 2500 rpm for 15 minutes. The supernatant containing the DNA was gently transferred to another 15 mL polypropylene tube and the tube with the precipitated protein pellet was discarded. Two volumes of room temperature absolute ethanol were added to

the supernatant and the tube inverted several times to precipitate the DNA. The DNA strands usually formed a small ball which floated to the surface and could be 'hooked' out with a nine inch glass pasteur pipette heated to form a hook at the end. This pasteur pipette was then held vertically in a test tube holder and rinsed carefully with a stream of 70% ethanol and air dried. The end with the DNA attached was removed in to a 1.5 mL Eppendorf centrifuge tube and 100-500 mL of TE (10.0 mM Tris, 1.0mM EDTA, pH8.0) added depending on a visual estimation of the amount of DNA.

3.2.2 Polymorphonuclear Cells

These cells were prepared by Laura Sampson-Murphy of the Immunology Research Laboratory by Ficoll-Hypaque separation of whole blood collected in ACD tubes. Each 7 mL ACD tube of blood yielded two Ficoll tubes of separated cells and from this two tubes of polymorphonuclear cells in Hanks or RPMI solution. Two to four tubes of cells in media were usually available from each person. These white cells were often greatly contaminated with red cells and with Ficoll-Hypaque. Because of this, special adaptations to the above red blood cell lysis procedure and salting out procedure explained in the previous section were carried out. Each tube was centrifuged at 1500 rpm for 10 minutes to pellet the cells and

then washed twice in saline. Ammonium chloride tris solution was added to fill the tubes after the last saline wash and the tubes were incubated for 15 minutes at 37 °C. After the red blood cell lysis, which was never as complete as when whole blood from EDTA tubes was used, the cells were spun as before at 2500 rpm. The cell pellet was resuspended in 5 mL of saline and the contents of two tubes were combined in one for the rest of the procedure. Although samples still appeared contaminated after this step, repeating the ammonium chloride tris step or another saline wash did not seem to improve the condition of the sample and often resulted in loss of white cells. From this point the DNA extraction procedure was the same as for whole blood.

The amount of DNA, estimated visually, in this procedure varied considerably from sufficient to hook out by the usual method to a few visible strands to none visible. In the last two cases the ethanol solution containing the DNA was centrifuged at 2500 for 15 minutes to pellet any small fragments of DNA at the bottom of the tube. The supernatant was aspirated and 1 mL of 70% ethanol was added. The tube was inverted several times but not vortexed. The supernatant was aspirated unless the pellet was loose. If it was, it was centrifuged again before aspiration, but this was very rare.

The DNA pellet in the tube was then left in the air to dry for approximately two hours before TE was added (200 µL). After leaving the DNA at room temperature in TE for about one hour, the tube was put on a rotator for one hour to continue dissolving the DNA in TE. It was then transferred to a 1.5 mL tube and stored at 4 °C.

3.2.3 EBV Transformed B Cells and Small Numbers of PBLs

In those cases where the sources of DNA were large numbers of EBV transformed B cells, the salting out procedure was still followed as described for whole blood except the initial red cell lysis step was omitted. Cells in media were pelleted by centrifugation (5 minutes at 2500 rpm) and washed twice with saline. From this step, the procedure was the same as with whole blood.

There was not always a great deal of sample material from which to extract DNA. Thus, the procedure had to be adjusted accordingly. Both the West Coast family samples and some of the SLE samples were stored at -70°C or in liquid nitrogen in tubes containing 1 mL of PBLs or EBV transformed B cells in freezing solution at 5,000,000 cells/mL. Because of the low amount of DNA that would be produced from this smaller number

of cells, the volumes were changed and the extractions done in 1.5 mL tubes.

The PBLs or B cells were thawed at room temperature, immediately put into 1.5 mL Eppendorf tubes and centrifuged in an Eppendorf centrifuge at maximum rpms for five minutes to pellet cells. The supernatant was aspirated and the cell pellet was resuspended in 1 mL of saline solution and centrifuged for five minutes to pellet cells. The cell pellet was resuspended in 300 µL of nucleic lysis buffer to which 20 µL of 10% SDS solution and 12.5 µL of pronase E (20 mg/mL) were added and incubated for one night at 37°C or for the other times previously indicated. After digestion, 100 µL of saturated sodium chloride solution was added, and the tube centrifuged for five minutes. The supernatant was pipetted into a fresh tube, and, if cloudy, spun again (extra sodium chloride was not added) and the supernatant removed to another fresh tube. Then 700 µL of 95% ethanol were added to the supernatant and the tube inverted while the DNA precipitated. The DNA strands were not always visible but a small pellet could usually be seen when the tube was centrifuged for approximately 30 seconds. The supernatant was removed and the pellet washed with 1 mL of 70% ethanol. The mixture was not

vortexed. If the pellet was loose, the tube was centrifuged again for 30 seconds and in both cases the supernatant was emptied. The pellet was allowed to dry for approximately 15-20 minutes and then 100-400 µL of TE was added. All DNA was stored at 4°C.

3.3 Amplification of the TNFa Microsatellite

The methodology was adapted from that used by Jongeneel and colleagues (1991).

DNA primers required for the specific amplification of the TNFa microsatellite were manufactured by Oligos Etc. (Bethel, ME) with the following sequences:

IR1 5'-GCACTCCAGCCTAGGCCACAGA-3'

IR2 5'-GCCTCTAGATTTCATCCAGCCACAG-3'

IR4 5'-CCTCTCTCCCCTGCAACACACA-3'

The amplification of the TNFa microsatellite was carried out in two separate polymerase chain reactions (PCRs). The first amplification was carried out in a final volume of 20 µL with 1 µL of genomic DNA of unknown concentration and a final concentration of 1 µM for primers IR1 and IR2, 0.25mM of dATP, dCTP, dGTP, dTTP (all nucleotides from Gibco/BRL), 1.5mM $MgCl_2$, 1X Taq DNA Polymerase Reaction Buffer (both $MgCl_2$ and

buffer supplied with the polymerase), and 0.05 U/μL of Taq DNA Polymerase (Promega, Madison, WI).

The genomic DNA (in distilled water) was denatured at 95 °C for five minutes in the Perkin Elmer Cetus DNA thermocycler. The remaining ingredients were then added, the mixture vortexed, and briefly centrifuged to collect the tube's contents. Mineral oil (Sigma) was overlaid on top to help prevent evaporation during the amplification cycles which consisted of 20 cycles, each one 94 °C for 25 seconds, 58 °C for 60 seconds and 74 °C for 60 seconds.

In the second amplification, 7.8 μM of the internal primer IR4 and 0.25 μM of radiolabelled IR4 in a final volume of 2 μL buffer containing 0.25 mM dNTP mix, 1X Taq DNA polymerase buffer, 1.5 mM $MgCl_2$ and 0.5 U/μL of Taq DNA Polymerase were added to the contents of the previous amplification. This mixture was then subjected to the same amplification cycles as in the first amplification. The radiolabelled primer was end-labeled with gamma phosphorous-32 (Amersham, Oakville, ON) using T4 polynucleotide kinase (Gibco/BRL). To label enough primer for one reaction 0.05 μL of 10 pmol/μL of IR4 was added to 0.05 μL of kinase 10X

buffer, 0.07 µL of distilled water, 0.3 µL of gamma phosphorous-32, and 0.03 of polynucleotide kinase enzyme.

3.4 Denaturing Gel Electrophoresis

PCR products were analyzed on a 41 cm long, 36.5 cm wide, 0.35 cm thick denaturing gel (sequencing gel) consisting of 7% polyacrylamide (BioRad), 5.7 M urea (Gibco/BRL) and 1X TBE. Gels were generally prepared the day before they were run but were, on occasion, used the same day or two days later without any adverse effects.

Samples (2 µL aliquots) were treated with 1 µL formamide dye (0.1% xylene cyanol [BioRad], 0.1% bromophenol blue [BioRad], 10 mM EDTA (pH8) in formamide [BDH]), heated at 95 °C for 8 minutes and immediately placed on ice just prior to being loaded on the denaturing gel.

Gels were run at 60-75 watts (W), constant power, for 2-3 hours, until the xylene cyanol dye front was in the last quarter of the gel. After completion, 3 MM Whatman filter (Fisher, Dartmouth, NS) paper was used to 'peel' the gel off the glass plate. It was subsequently dried for 2 hours at 80 °C. The radioactively labeled bands were visualized by

autoradiography using Kodak Scientific Imaging Film (XAR 5) and variable exposure times.

3.5 Assignment of Alleles/Sizing of PCR Generated Fragments

Alleles produced by microsatellite specific PCR amplification and visualized by separation of DNA fragments on denaturing polyacrylamide gels and autoradiography were assigned numbers in accordance with their size (Jongeneel et al., 1991). M13mp18 bacteriophage DNA was sequenced by the chain termination method according to the manufacturer's protocol of the Sequenase Version 2.0 Sequencing Kit (United States Biochemical Corporation). The sequenced bacteriophage DNA was run on the same gel as PCR fragments to assess the size of the bands produced by PCR amplification. No person had more than two major bands, although sometimes only one or none was visible. Although only a maximum of two major bands was visible, several lighter, usually smaller, fragments could also be seen.

Alleles, represented by major bands, were given numbers based on their size (in base pairs) with 1 representing the smallest allele and 14 the largest. To further test the agreement of the typing with published nomenclature, several workshop cell lines, previously typed by Jongeneel et al.

(1991) in one of the definitive papers on this microsatellite, were typed as part of this project.

3.6 Within and Between Run Reproducibility

To allow for comparison between different runs, a standard set of samples was established which was used on every gel. It consisted of DNA from three heterozygous individuals. DNA samples from these people were mixed together either before or after PCR. Both methods were effective, but using a mix of the DNA before amplification proved to be more efficient. A standard stock solution of combined DNA from the three individuals was made and aliquots taken for amplification for each set of PCR reactions. Because equal aliquots from this same standard stock solution could be used on each gel, this allowed for more consistent typing. Only one lane was used for the standard solution, and, therefore, it could be repeated across the width of the gel at specific intervals. In addition, a person of known heterozygous type with consecutive alleles (differing by only two base pairs) was also run on each gel as a reference for the standards and as a means of examining the banding pattern of two consecutive alleles on different gels.

As a further means of establishing reproducibility, a family study was undertaken to check for reliability and consistency in naming alleles. Also random individuals were done more than once and scored separately on different gels over the months of typing. These results are shown in the results section.

3.7 HLA Typing

HLA-A,-B,-C, and -DR typing was done using standard complement mediated cytotoxicity assays in the laboratories where the samples were collected for other projects. Access to the results were made available for use in this project. Hong and coworkers did some of the HLA-DR typing on their Korean samples using molecular techniques outlined in their paper (Hong et al., 1994).

HLA-DR typing for the Newfoundland Caucasian SLE population was performed as part of this project using a polymerase chain reaction-sequence specific primer (PCR-SSP) based assay purchased from One Lambda, Inc. The protocol provided by the manufacturer was followed exactly. PCR products derived from this assay were run on 2% agarose gels in 0.5X TBE for approximately 15 minutes at 120 V or until the red tracking dye had migrated approximately 1 cm into the gel.

The DNA bands were visualized by ultraviolet illumination. Positive lanes had DNA bands between the primer and internal control bands. The results were interpreted using the worksheet provided with the kit. The Programmable Thermal Blok II from LAB-LINE was used for the PCR amplification. For a few SLE samples, only serological data previously acquired was available.

3.8 Statistics

Comparisons between groups was done using contingency tables and Fisher's Exact Test using the statistical software InStat.

CHAPTER 4: RESULTS

4.1 Establishment of TNFa Microsatellite Typing

The first objective of this project was to establish the methodology for accurate and reliable typing of the TNFa microsatellite as described by Jongeneel and coworkers (1991) for use in this laboratory. This involved assigning alleles compatible with the numbering system used by Nedospasov and colleagues (1991) and Jongeneel and colleagues (1991), and checking the reliability and consistency of the data obtained. These results are detailed below.

4.1.1 Mendelian Inheritance of TNFa Alleles

Several small nuclear families were analysed to demonstrate Mendelian inheritance of this microsatellite. Two such families are illustrated in Figure 4.1, but all gave comparable results showing segregation of the microsatellite sizes in Mendelian fashion with stably transmitted alleles.

4.1.2 TNFa Allele Assignment with Current Nomenclature

Amplification of the TNFa microsatellite produced one or two major bands per person, often accompanied by shadow bands in a ladder effect (Figure 4.2.). Normally, the major

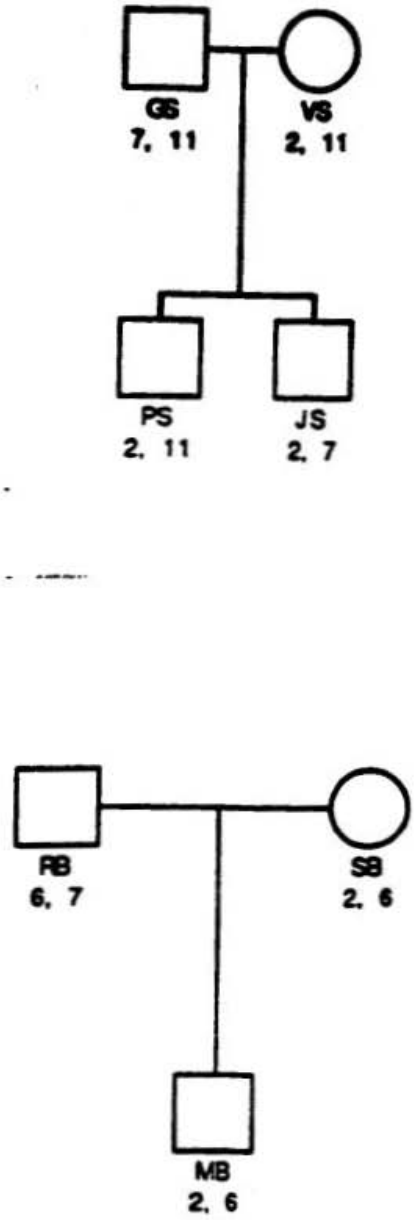

Figure 4.1: Mendelian inheritance of microsatellite TNFa in two small nuclear families. Initials identify individuals in the family and numbers represent TNFa alleles. Squares=males; circles=females.

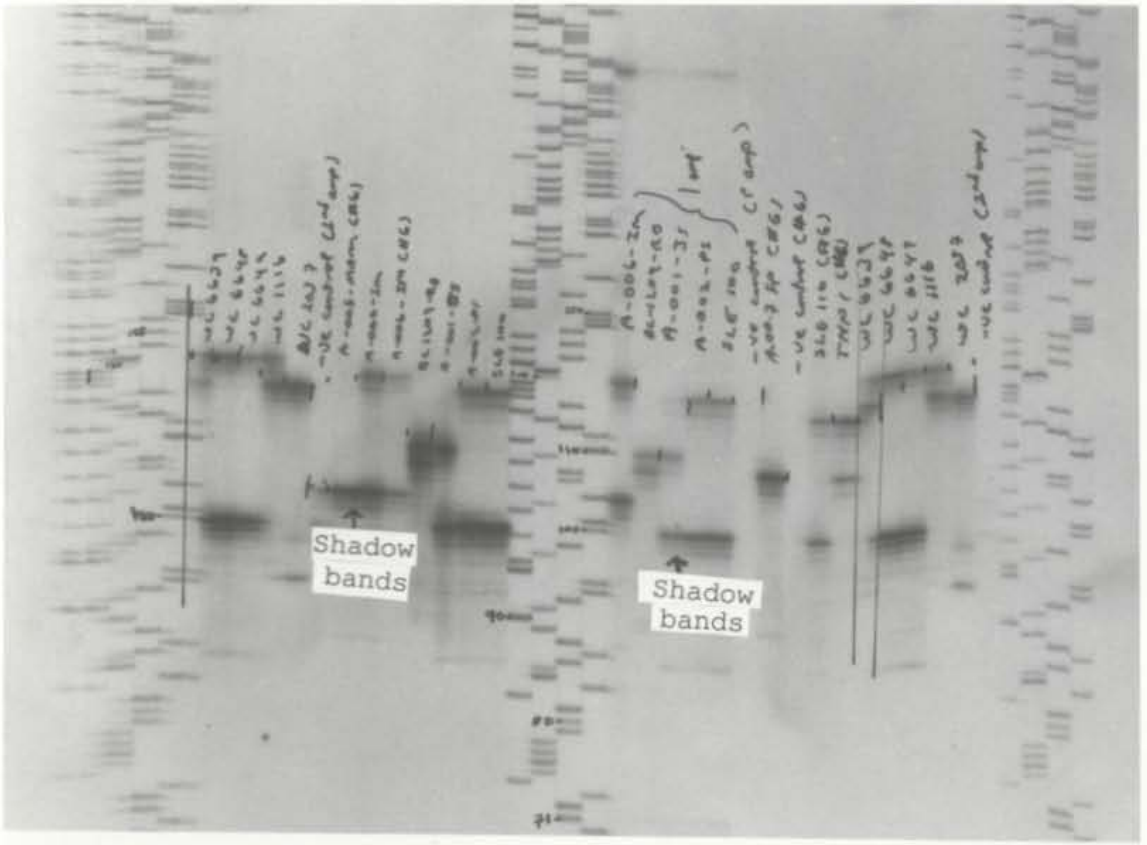

Figure 4.2: Shadow bands produced by amplification of the TNFa microsatellite.

(darkest) band occurred first followed by the shadow bands starting two base pairs behind. This is a well documented effect of PCR of microsatellites (Nedospasov et al. 1991) and did not interfere for the most part with the typing of alleles, except sometimes in the case of consecutive alleles, i.e., alleles differing by one repeat unit, in this case two base pairs (Figure 4.3.).

Bands representing the different variants of this microsatellite were given consecutive numbers for consecutive alleles with 1 representing the smallest variant and 14 the largest (Table 4.1). Because the same naming system was being used as published in the definitive papers on this subject, it was important to assess whether the numbers for the variants corresponded to those published. Two factors were involved in this assessment:

1) originally, sequenced M13mp18 bacteriophage DNA was used to find the actual size of the allele which was compared to published sizes.

2) workshop cell lines typed as part of this thesis were compared with the results obtained by the original investigators in this field.

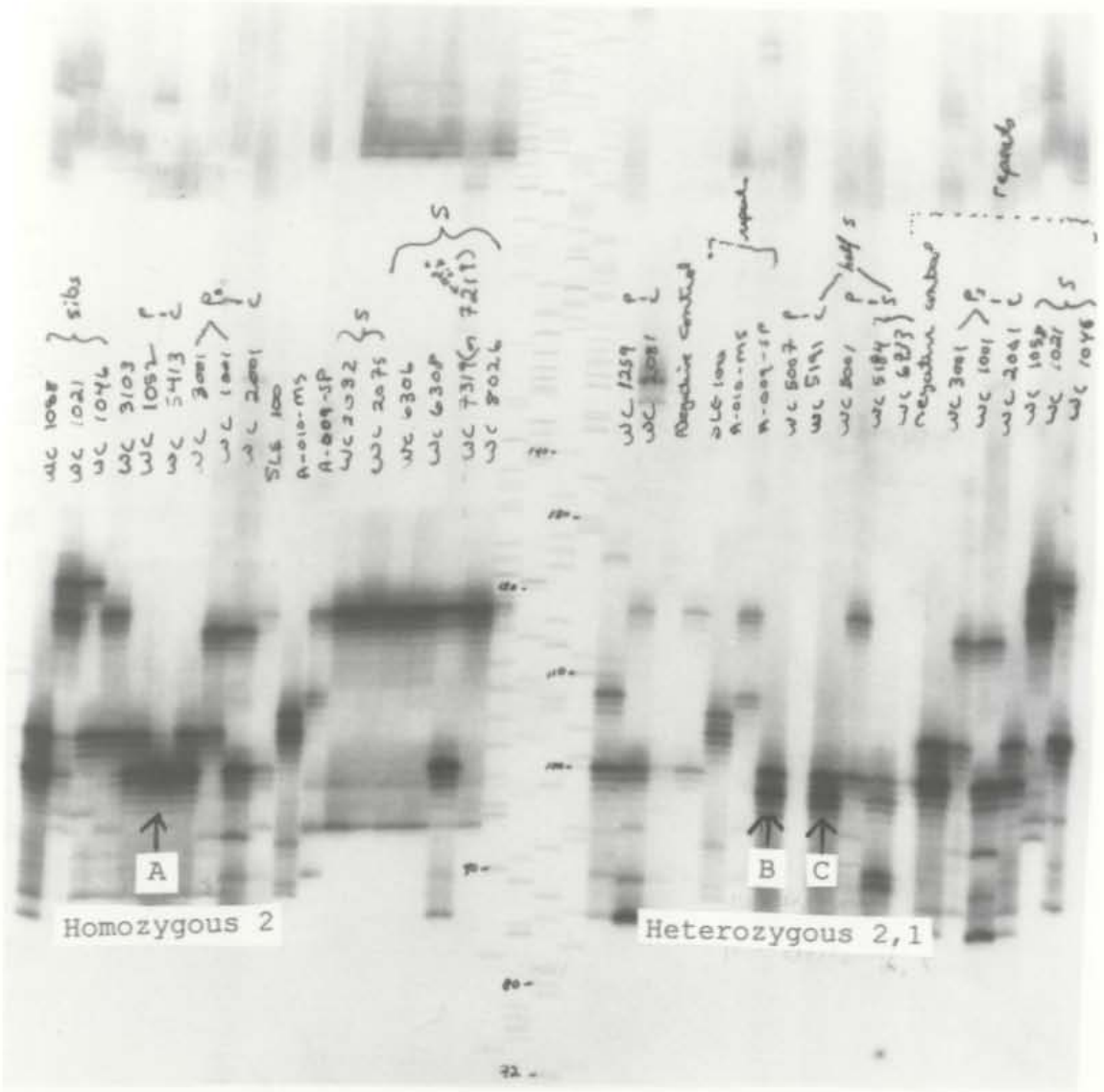

Figure 4.3: Homozygosity and heterozygosity for consecutive alleles in TNFa microsatellite typing. Individual A is homozygous for allele 2. Both individuals B and C have alleles 2 and 1.

Table 4.1: Designation of alleles of microsatellite TNFa based on size of amplified DNA fragment

Allele Number	Length of Amplified DNA (bp)	Number of CA repeats
1	97	6
2	99	7
3	101	8
4	103	9
5	105	10
6	107	11
7	109	12
8	111	13
9	113	14
10	115	15
11	117	16
12	119	17
13	121	18
14	123	19

4.1.2A Sizing of alleles

Comparison of sequenced M13mp18 DNA with microsatellite fragments did give the approximate size of the variants. However, it produced two difficulties. First, the bands of the amplified fragments did not always exactly correspond to that of the ladder and ½ sizes were often the result. Also, the farther from the lanes of sequenced bacteriophage DNA the sample lanes were, the more difficult it became to accurately and easily tell the size. Secondly, four consecutive lanes were required for the running of the sequenced DNA, one lane for each nucleotide. Thus, space did not permit repetition of these markers on the gel. This was especially a concern because, sometimes, samples at the end "sloped" down or up and made it difficult to determine allele size based on running sequenced M13 in the middle four lanes.

4.1.2B Workshop Cell Lines

The results of thirteen MHC homozygous workshop cell lines typed in his lab were kindly provided by Dr. Victor Jongeneel, an author of one of the definitive papers on this microsatellite (Jongeneel et al. 1991). Seven of these had been previously typed as part of this thesis and the results agreed, thus showing naming of alleles was consistent with that published (Table 4.2.).

Table 4.2: TNFa typing of previously published workshop cell lines

Workshop Cell Line	TNFa Type
KAS011	10
MZ070078	2
RSH	6
SWEIG	10
TAB 089	6
WT47	4
WT51	4

4.1.3 Accuracy and Consistency of TNFa Typing

To help with allele assignment and consistency between runs, a panel of mixed DNA was used as a marker. The mixed DNA consisted of DNA from a number of individual samples that had been typed and alleles assigned based on the methods referred to in the preceding sections; i.e., alleles were assigned by using sequenced M13mp18 DNA and by comparison with the alleles of the workshop cell lines. For each PCR and gel that was run, an aliquot of premixed samples was taken and amplified. Two different panels were used over the course of the typing. The second panel, which was used for all later typing, provided a more even spread of alleles (Table 4.3.). Also included in each PCR and gel was DNA from one particular individual who was heterozygous for consecutive alleles (TNFa4/TNFa5).

Once methodology and allele nomenclature were established, the accuracy and reliability of allele assignment were tested by:

1) typing repeat samples on different gels

2) examining the consistency of typing results from members of a large family sharing MHC haplotypes.

Table 4.3: Individual DNA in two different panels used as markers for TNFa typing

	Individual	TNFa Type
Original Panel	A-010-MS	4, 5
Alleles: 2,4,5,6,7,11,12	BC-1209-RB	6, 7
	BC-1210-SB	2, 6
	A-006-IM	4, 12
	A-008-BP	6, 11
Second Panel	SLE 220	6, 8
Alleles: 2,4,6,8,10,12	A-006-IM	4, 12
	SLE 110	2, 10

4.1.3A Between Run Reliability

Of the over 500 samples typed, 73 were retyped, either from the same tube of DNA or, in a few cases, from different tubes of DNA extracted from the same person. These were done randomly over the course of several months. The samples were amplified, run on gels and scored from the autoradiographs without knowledge of the previous results. There were 17 homozygotes, 6 heterozygotes with alleles differing by only two base pairs and 50 heterozygotes with alleles differing by more than two base pairs. In no case were completely differing results assigned during the second typing. The only discrepancies were in distinguishing heterozygosity with two consecutive alleles from homozygosity of the larger allele. There were two cases where a homozygote was given a questionable homozygote status for the second typing. Questionable homozygote status was a way of denoting an individual who was most likely homozygous, but might be, although much less likely, a heterozyote for two consecutive alleles, the larger one as in the homozygous typing and the next smaller one. There was one case of the reverse being true. The initial typing of a homozygote was questionable (although most likely), and, when retyped, it was classified as a homozygous.

4.1.3B Analysis of Family Data for Consistency in Typing

Over 300 samples from a large Newfoundland kindred, previously studied in the Immunology Laboratories of Memorial University of Newfoundland, was available for use in this study. The extensive pedigree contained MHC haplotypes from three generations on HLA-A, HLA-B, Bf, and C4. The original analysis only contained information of HLA-A, HLA-B, and Bf (the C4 study occurred later using stored samples). After extensive interviews with family members and analysis of available records, the original three-loci haplotypes were given "entry numbers" as a way of indicating whether MHC haplotypes with the same combination of alleles could be identified as being inherited from the same person. Independent examples of the same haplotype from apparently unrelated individuals were given different entry numbers for that haplotype, i.e., these were unrelated examples of the same combination of alleles at the MHC loci studied. Individuals with the same MHC haplotype inherited from the same ancestor were all given the same entry number for that haplotype. The C4 data were obtained at a later time and added to the haplotypes. This study provided TNFa alleles for various family members which were then assigned to the already existing haplotypes. Although for some haplotypes used in this study there was only one individual to represent that particular haplotype, for many of the haplotypes there were

several representatives with that same portion of the chromosome.

Individuals were typed as either homozygous or heterozygous for TNFa microsatellite variants. Identification of heterozygous individuals in which the microsatellite variants were more than two base pairs apart in size was the simplest situation. No detectable errors either in retyping or when comparing family data were evident in this group. Four other possible groupings existed and were examined in the West Coast family data. Individuals were identified as definite homozygotes, questionable homozygotes, definite heterozygotes for consecutive alleles, or questionable heterozygotes for consecutive alleles.

There were 303 individuals typed from this family material, many, but not all, of whom shared haplotypes with each other. Of these 303 individuals, there were 12 detectable instances of disagreement with pedigree and MHC data. Of the twelve discrepancies, a case of mislabeling of the DNA tubes seemed to be most likely for four of the individuals. This is not surprising as numbers had been written directly on the tubes and in some cases were a little worn. Also, these numbers were then transcribed to other tubes which could have resulted in an error. Five cases had, upon closer examination, conflicting family data or disagreement between different records, and were excluded from

the analysis.

The remaining three were incorrectly typed as most likely heterozygous for consecutive alleles (questionable heterozygotes) when family data and reexamination of the autoradiographs showed instead homozygosity for the larger allele. One of these results had been noted as possibly being contaminated from the loading of a neighbouring sample during gel electrophoresis. Exclusion of this sample because of possible contamination (instead of mistyping) leaves only two individuals with clear evidence of typing errors, and both fall into the category of questionable heterozygotes (Table 4.4.) of which 14 individuals were classified in total. None of the other categories showed any evidence of disagreement with family data.

4.2 Allele and Haplotype Associations of TNFa with MHC Loci in an Extended Family

The large Newfoundland family provided an excellent source of material for haplotype analysis of TNFa microsatellite variants with HLA-B and complotype (the combined haplotype of the genes for the components of complement found in the Class III region of the MHC and inherited together on the same chromosome). In this case, complotype did not include C2, only Bf, C4A and C4B. The notation used is written in abbreviated format in that order.

Table 4.4: Distinguishing between homozygotes and heterozygotes in a large kindred

Category	No. of people typed	No. of people with family data disagreement
Definite Homozygotes	60	0
Questionable Homozygotes	6	0
Definite Heterozygotes (for consecutive alleles)	19	0
Questionable Heterozygotes (for consecutive alleles)	14	2
Definite Heterozygotes (for non-consecutive alleles)	194	0
Excluded	10	
Total	303	2

For example, S01 represents BfS, C4AQ0, C4B1.

The 293 people with usable TNFa types (303 people minus ten that were excluded--see preceding section) from this large extended kinship yielded 120 independent haplotypes that were not identical by descent, that is, were not inherited from a common ancestor as ascertained from the family studies. Information was available for TNFa and HLA-B for all 120 haplotypes. However, information for Bf, C4A and C4B was only available for 114 of these haplotypes, and, of these, fully informative C4 haplotyping was only available for 75. This was because in 39 haplotypes the family material available was not fully informative for the C4 typing and null alleles (Q0) at one or both of the C4 loci could not be excluded. This is indicated in these haplotypes by an asterisk (*) next to the alternative allele, usually A3 or B1. Therefore, an A3* means the individual is either A3 or AQ0 at that locus. Similarly, B1* means an individual is either B1 or BQ0 at that locus.

4.2.1 Association of TNFa Variants with Individual HLA-B and Complotype Alleles

In this extended family, 10 alleles were observed for the microsatellite TNFa. TNFa2 was the most frequent allele followed by a11, and a6 in that order. These three alleles represented 69% of the TNFa allelic variation observed in this data set. There were four alleles (a1, a5, a9, and a13) that

occurred at a frequency of less than five percent each.

Sixteen HLA-B alleles were identified in this population with B7, B12, B8 and B35 being the most common HLA-B alleles in that order. There were also three alleles (B39, B53, B64) that only occurred once each in this population of chromosomes. Of the 14 complotypes observed, S01, S31, S3*1 and S30 were the most common.

4.2.1A TNFa and HLA-B Allelic Associations

For the HLA-B and TNFa allelic associations (Table 4.5), it can be observed that certain TNFa alleles occur with a greater variety of HLA-B alleles than others. However, this is not necessarily a characteristic of those TNFa alleles of highest frequency. For example, although TNFa2 has the highest frequency and occurs with the greatest number of HLA-B alleles, TNFa11 with the next highest count (27) occurs with only four HLA-B alleles, whereas TNFa6, observed 18 times, was found together with seven different HLA-B alleles. TNFa10, occurring in 10 unrelated chromosomes, was found with 5 different HLA-B alleles. Nevertheless, several associations can be seen in this data. First, all 19 HLA-B8 haplotypes were observed with TNFa2 and no other microsatellite variant, although TNFa2 itself was seen with a variety of HLA-B alleles (9). HLA-B7 was strongly associated with TNFa11 (19/22) as was HLA-B 40 (6/8). HLA-B12 was associated with both TNFa4

Table 4.5: Number of independent chromosomes containing various combinations of alleles at the HLA–B and TNFa loci

	TNFa													
HLA–B	1	2	3	4	5	6	7	8	9	10	11	12	13	Total
5		3				1				1				5
7		1					2				19			22
8		19												19
12				10		8	1				1			20
14		3												3
15		7				1								8
16										2	1			3
18	4	1								4				9
22										2				2
27						2	1							3
35		2		1	2	4	3			1				13
37									2					2
39													1	1
40		1				1					6			8
53						1								1
64		1												1
Total	4	38	0	11	2	18	7	0	2	10	27	0	1	120

(10/20) and a6 (8/20). It is interesting to note that although 11 examples of TNFa4 were observed, 10 occurred with HLA-B12. Conversely, there were HLA-B alleles that did not seem to show strong association with any one allele, HLA-B35 being the most obvious. It was observed with six different TNFa alleles even though there were only 13 counts of HLA-B35 in total.

4.2.1B TNFa and Complotype Allelic Associations

Examination of Table 4.6 (on pp 68-69) of TNFa and complotype associations yields similar results. The complotype S01 is associated almost exclusively with TNFa2 (17/19). However, the reverse is not true. TNFa2 is observed with a variety of other complotypes. Also, although S31 is seen with several TNFa alleles, at least half were observed with TNFa11. Only eight examples of the S42 complotype were TNFa typed and four different TNFa alleles were observed with that complotype.

4.2.2 MHC Five-Loci Haplotype Analysis

Analysis of the complete haplotypes available allowed identification of independent examples of haplotypes carrying the same combination of alleles.

Table 4.6: Number of independent chromosomes containing various combinations of complotypes and TNFa alleles

Complo–type	TNFa													Total
	1	2	3	4	5	6	7	8	9	10	11	12	13	
S01	1	17		1										19
S02		1												1
S121						1	1							2
S21										1				1
S2(2,1)		1												1
F130	2													2
S30		2		1		5	2							10
F30				6	1									7
F31		1				3								4
S31		3		2		1				2	9			17
S33		1												1
F(3,2)0													1	1
S42		3				2				2	1			8
S44		1												1
S31*		2								1	4			7
S3*1		3				1	1		1	2	9			17
S3*1*						1			1		4			6
F31*				1		1								2
F3*1					1	2								3
F3*1*						1	1							2

Table 4.6: Number of independent chromosomes containing various combinations of complotypes and TNFa alleles

	TNFa													
S3*3		1												1
S61*		1												1
Total	3	37	0	11	2	18	5	0	2	8	27	0	1	114

*means allele as written or Q0

4.2.2A Common MHC Haplotypes

Four allele haplotypes of HLA-B, Bf, C4A and C4B were analyzed for possible TNFa associations. Any combination of alleles at the five loci that occurred more than once in the 75 complete unrelated haplotypes available were considered extended haplotypes in this population. Ten such haplotypes were observed (Table 4.7), each occurring at least twice. In some cases, independent haplotypes had the same HLA-B, Bf, C4A and C4B alleles but not the same TNFa allele. There were five examples of this (Table 4.8) where at least two independent haplotypes were identical for all alleles except the TNFa microsatellite.

The raw data were also analyzed for the presence of previously reported, well characterized extended haplotypes, often referred to by many investigators as ancestral haplotypes. Their occurrence with specific TNFa alleles and their frequency in this data set were determined (Table 4.9). Although HLA-DR data were not available for this study, ancestral haplotypes are normally characterized by allele combinations at HLA-B, complotype and HLA-DR, and are named according to the HLA-B allele. The most common ancestral haplotype in this population is AH 8.1 (HLA-B8;BfS;C4AQ0,C4B1) with 15 independent examples. A comparison of Table 4.7 with these extended haplotypes indicates that in the data set used for this thesis there are five haplotypes that may be

Table 4.7: Extended haplotypes occurring more than once on unrelated chromosomes in the WC family

HLA-B	TNFa	Bf	C4A	C4B	No.
18	1	F1	3	0	2
8	2	S	0	1	15
14	2	S	0	1	2
15	2	S	3	1	2
12	4	F	3	0	5
12	4	S	3	1	2
12	6	S	3	0	5
7	7	S	3	0	2
7	11	S	3	1	5
40	11	S	3	1	4

Table 4.8: Haplotypes carrying the same HLA–B, Bf, C4A, and C4B alleles but different TNFα variants

HLA–B	Bf	C4A	C4B	TNFα	No.
5	S	4	2	2	1
				6	1
12	S	3	0	6	5
				4	1
35	F	3	0	4	1
				5	1
35	S	3	1	6	1
				10	1
40	S	3	1	11	4
				2	1

Table 4.9: TNFα alleles on independent examples of ancestral haplotypes

	Ancestral Haplotype					
AH*	HLA–B	Bf	C4A	C4B	TNFα allele[a]	No. individual examples[b]
7.1	7	S	3	1	11	5
8.1	8	S	Q0	1	2	15
44.1	12	S	3	Q0	6 (4)	5 (1)
44.3	12	S	Q0	1	4	1
65.1	14	S	2	1+2	2	1
62.1	15	S	3	3	2	1
38.1	16	S	2	1	10	1
18.1	18	S	4	2	10	1
18.2	18	F1	3	Q0	1	2
18.3	18	S	3	1	10	1
35.1	35	S	3	1	6 (10)	1 (1)
35.3	35	S	3	Q0	2	1
60.1	40	S	3	1	11 (2)	4 (1)

[a]number in brackets indicates alternative allele associated with this haplotype in this data set.
[b]number in brackets indicates number of individual examples of alternate allele with this haplotype
*AH=ancestral haplotype. Ancestral haplotypes are named for the HLA–B allele and have been taken from Degli–Esposti et al., 1992a and Degli–Esposti et al., 1995.

considered extended haplotypes in this population that have not been well documented as part of the ancestral haplotype sets in the literature. These are (written as HLA-B;TNFa;Bf;C4A;C4B): B7,a7,S30; B12,a4,F30; B12,a4,S31; B14,a2,S01; B15,a2,S31.

4.2.2B TNFa Associations with Haplotype Fragments

The 75 complete haplotypes were also used to analyze whether the TNFa microsatellite alleles were more often associated with the HLA-B fragment or with the C4 fragment of the specific ancestral haplotype. In this study, complete ancestral haplotypes have characteristic alleles at the HLA-B locus and the three complement loci. A fragment of an ancestral haplotype is defined as a segment, say B-TNF or TNF-Bf-C4, containing the alleles defining an ancestral haplotype but with different alleles at the rest of the haplotype. For example, of the 30 occurrences of the TNFa2 allele, there were 18 incidences of the full or partial 8.1 ancestral haplotype occurring with this allele. Fifteen of these occurred with the full HLA-B--complotype haplotype of ancestral haplotype 8.1 (Table 4.10). However there was also one occurrence of TNFa2 occurring on a haplotype containing the HLA-B8 allele (of the 8.1 AH) but with a different complotype (not S01 of the AH 8.1). Similarly, there were two occurrences of TNFa2 with the S01 complotype but without the HLA-B8.

Table 4.10: Association of TNFα alleles with ancestral haplotypes and HLA–B and complotype fragments

		Ancestral Haplotype					B–TNF fragment[a]		TNF–complotype fragment[b]	
TNFα allele	Total No.	HLA–B	Bf	C4A	C4B	No.	HLA–B	No.	Complo–type	No.
1	3	18	F1	3	0	2	18	1	F102	0
2	30	8	S	0	1	15	8	1	S01	2
		14	S	2	1+2	1	14	2	S2(1,2)	0
		15	S	3	3	1	15	2	S33	0
		35	S	3	0	1	35	1	S30	1
		40	S	3	1	1	40	0	S31	2
4	10	12	S	0	1	1	12	7	S01	0
		12	S	3	0	1	–	–	S30	0
6	12	12	S	3	0	5	12	2	S30	0
		35	S	3	1	1	35	1	S31	0
10	5	16	S	2	1	1	16	0	S21	0
		18	S	4	2	1	18	0	S42	1
		18	S	3	1	1	–	–	S31	0
		35	S	3	1	1	35	0	–	–
11	10	7	S	3	1	5	7	1	S31	0
		40	S	3	1	4	40	0	–	–
Total						42		18		6

[a]The B–TNF fragment represents haplotypes that contain the HLA–B allele and TNFα allele indicated for that ancestral haplotype but have a different complotype than the one associated with that ancestral haplotype.

[b]The TNF–complotype fragment represents haplotypes that contain the complotype and TNFα allele indicated for that ancestral haplotype but have a different HLA–B allele than the one associated with that ancestral haplotype.

The results (Table 4.10) indicate that the extended haplotype comprising the five markers used in this analysis, HLA-B, TNFa, Bf, C4A and C4B-the last three of which are written together as a complotype-occurs more often than fragments of the haplotype. When fragments of known ancestral haplotypes do occur, the TNFa variant occurs more often with the characteristic HLA-B allele of that haplotype than with the characteristic complotype. In 18 fragments of extended haplotypes, the TNFa allele occurred with the characteristic HLA-B allele (associated with that extended haplotype) but with a different complotype, and in 6, it occurred with the complotype but a different HLA-B allele.

In summary, analysis of the family data demonstrated considerable variety in the TNFa variants that occurred with particular HLA-B or complotype alleles. However, of the associations that did exist, the most obvious were between TNFa and HLA-B alleles. Ancestral haplotypes were identified in the population and TNFa alleles occurred most often with full ancestral haplotypes. However, some haplotypes represented possibly rare ancestral haplotypes or ancient recombinations between ancestral haplotypes. In these cases, TNFa alleles occurred more often with the HLA-B fragment of a defined ancestral haplotype and a different complotype than with the associated complotype and a different HLA-B allele. There were also examples in this population of haplotypes

carrying the same alleles at the HLA-B,Bf,C4A and C4B loci but having different TNFa alleles, even though the TNFa microsatellite occurs between the HLA-B and the complement loci.

4.3 TNFa and Null alleles of the Fourth Component of Complement

There are two isotypes of the fourth component of complement: C4A and C4B, encoded by closely linked genes in the MHC. C4A and C4B have a variety of allelic forms, one of which is a null allele. Null alleles are defined as those for which no protein is produced and are designated Q0 (quantity zero). The assignment of null alleles may be done through informative family studies but it has also been done using densitometry by comparing the relative intensities of the C4A band(s) to the C4B band(s) after the bands are separated electrophoretically on typing gels. This section examines whether any TNFa alleles are associated with the null alleles of either C4A or C4B.

4.3.1 Null Alleles Assigned by Family Analysis

As reported in Section 4.2, the large extended family yielded 75 haplotypes that were completely informative for the C4 loci. Of these, 20 were C4AQ0 occurring as part of 5 different haplotypes (Table 4.11). Three of these haplotypes

Table 4.11: TNFa alleles on haplotypes carrying C4AQ0 in an extended family

Haplotype (HLA-B;Bf;C4A;C4B)	TNFa Allele	No. of unrelated examples
18;S;0;1	1	1
8;S;0;1	2	15
14;S;0;1	2	2
12;S;0;1	4	1
5;S;0;2	2	1

Table 4.12: TNFa alleles on haplotypes carrying C4BQ0 in an extended family

Haplotype (HLA-B;Bf;C4A;C4B)	TNFa allele	No. of unrelated examples
18;F1;3;0	1	2
18;S;3;0	2	1
35;S;3;0	2	1
12;S;3;0	4	1
12;F;3;0	4	5
35;F;3;0	4	1
35;F;3;0	5	1
12;S;3;0	6	5
7;S;3;0	7	2
39;F;(3,2);0	13	1

occurred with TNFa2 but the remaining two haplotypes each had a different TNFa allele.

There were also 20 incidences of C4BQ0 in this data set, representing 10 different haplotypes (Table 4.12). There was a total of seven different TNFa variants occurring with the C4BQ0 allele in this group.

4.3.2 Null Alleles Assigned Using Densitometry

Two groups, one normal and the other a SLE population, had C4 null alleles assigned using densitometry (Hong et al., 1994). Because this was not a family study, no TNFa--C4 haplotypes could be determined. However, it was apparent that in neither group was any one particular variant present in all people with a C4AQ0 allele defined by densitometry. Because TNFa2 did occur in 3 of the 5 haplotypes that had C4AQ0 assessed in the family study (see previous section), the numbers of individuals who possessed both TNFa2 and C4AQ0 in these two groups were determined (Table 4.13). The TNFa allele that occurred most frequently in individuals that carried a C4AQ0 was TNFa6, for both the normals with C4A nulls (7/10) and the SLE patients with C4A nulls (6/19). Many other TNFa variants also occurred in the C4A null individuals but TNFa6 occurred most often. No C4BQ0 allele was identified in the SLE patient group and only 2 individuals were assigned C4BQ0 in the normal population. Both these two individuals did

Table 4.13: TNFa alleles occurring in Korean individuals carrying a C4A null allele assigned by densitometry

TNFa allele	Number of individuals with TNFa allele	
	Normals with C4AQO (n=10)*	SLE with C4AQO (n=19)*
2	4	6
6	7	6

*n=number of individuals with C4AQO

share an allele, TNFa13.

4.4 TNFa Microsatellite in SLE

Systemic lupus erythematosus (SLE) is a complex, multifactorial disease. Several MHC associations with this disease exist and have been well documented, such as the increased frequency of the HLA-B8;BfS;C4AQ0;C4B1;DR3 haplotype in Caucasians. In section 4.2.2A, it was demonstrated that in this population the 8.1 extended haplotype is associated with the TNFa2 microsatellite allele. Therefore, it was the objective of this part of the project to determine if the TNFa2 allele was increased in the Newfoundland Caucasian SLE patient group. The contribution of this allele in the Korean SLE, not associated with the 8.1 ancestral haplotype, was also examined.

4.4.1 TNFa Alleles in Normal and SLE Populations

The distribution of TNFa alleles was determined in 109 healthy unrelated Caucasian individuals from Newfoundland (Table 4.14). This represented the normal allele frequency in the Newfoundland population. Microsatellite alleles TNFa1 through TNFa14 were present in this sample population but the frequency of the various alleles differed markedly from 27.5% for TNFa2 to 0.5% for a3, a8, a12 and a14, representing a

Table 4.14: TNFa microsatellite allele frequency in Korean and Caucasian normal and SLE populations

Allele	Korean				Caucasian			
	SLE n=96		Normal n=100		SLE n=54		Normal n=218	
	No.	%	No.	%	No.	%	No.	%
1	6	6.2	1	1.0	2	3.7	2	0.9
2	21	21.9	10	10.0	25	46.3	60	27.5
3	2	2.1	-	-	-	-	1	0.5
4	-	-	1	1.0	4	7.4	17	7.8
5	7	7.3	2	2.0	1	1.9	16	7.34
6	23	24.0	36	36.0	7	13.0	38	17.43
7	7	7.3	9	9.0	1	1.9	21	9.63
8	-	-	-	-	1	1.9	1	0.5
9	5	5.2	4	4.0	0	-	2	0.9
10	11	11.5	10	10.0	8	14.8	15	6.9
11	8	8.3	14	14.0	4	7.4	41	18.8
12	-	-	-	-	0	-	1	0.5
13	6	6.2	12	12.0	0	-	2	0.9
14	-	-	1	1.0	1	1.9	1	0.5

single occurrence of each. Similarly, 50 healthy, unrelated Koreans were typed (Table 4.14). There were only 11 TNFa variants in this sample population, the highest frequency occurring for TNFa6 at 36%.

Allele frequencies of 27 Newfoundland Caucasian and 48 Korean SLE patients were determined (Table 4.14). In the Newfoundland Caucasian SLE population, as in this normal Caucasian population, the TNFa2 allele was most frequent. Of the 27 Newfoundland Caucasian SLE patients examined, 70.4% had at least one TNFa2 allele compared with 52.3% of the normal population. Although the difference in the phenotypic frequency of TNFa2 between the two groups was not statistically significant, comparison by Fisher's Exact Test of the TNFa2 *allele* frequencies between the SLE and control groups (46.3% and 27.5% respectively, Table 4.14) did show that the increase in the SLE group was significant (p=0.009).

As in their normal population, the most frequent allele in the Korean SLE population was TNFa6, although this allele was less frequent than in the normal population (24% vs 36%). However, the TNFa2 allele frequency at 21.9% was significantly increased over the frequency of this allele (10%) in the Korean normal population (p=0.030). The difference in phenotypic frequency of TNFa2 between the Korean SLE and Korean normals was also significant (p=0.02).

4.4.2 TNFa2 and DR3

DR3 has also been reported in the literature to be associated with SLE in certain populations, especially Caucasian. Whether the TNFa2 allele occurred on the same haplotype as a DR3 in these disease populations could not be ascertained from this data because no family studies were done on the normal or SLE populations, and , therefore, no haplotypes could be derived. However, the number of TNFa2 individuals also carrying the DR3 allele for both SLE groups is shown in Table 4.15. TNFa2 and DR3 occur frequently in the same individual in the Caucasian SLE (the HLA-DR3 allele occurs in 13 out of 18 completely typed TNFa2 individuals). However, in the Korean SLE, only 2 individuals carry both the TNFa2 and DR3 allele (Table 4.15).

4.4.3 TNFa2 and C4AQ0 in Korean SLE

Information did not exist on C4AQ0 in the Newfoundland Caucasian SLE and normal populations used for this project. However, RFLP and densitometry were used by Hong and colleagues (1994) to identify null alleles of C4 in the Korean normal and SLE groups. Null alleles due to deletion were not detected. However, they reported in their paper an increase in C4AQ0, identified by densitometry, in their SLE population compared to their controls. The Korean SLE and Korean normals used in this project represented a non-selected subset of the

Table 4.15: Occurrence of the HLA-DR3 allele in SLE patients with TNFa2

	Number of TNFa2 patients with/without DR3 allele	
	Caucasian SLE (with TNFa2)	Korean SLE (with TNFa2)
HLA-DR3 positive	13	2
HLA-DR3 negative	5	17
Not typed	1	1
Total	19	20

original study population examined by Hong and colleagues. It was examined whether a significant increase of C4AQ0 was still present in this SLE population. Of 43 SLE patients with C4 types, 19 carried a C4AQ0. In the normals, 10 had a C4AQ0 out of a total of 46 (Table 4.16). This phenotypic difference was significant (p=0.041). As reported in Section 4.4.1, there was a significant increase in the phenotypic frequency of TNFa2 in the Korean SLE as well (Table 4.16).

There was a significant increase in the phenotypic frequency of both C4AQ0 and TNFa2 in the Korean SLE group compared to the control population. To examine if a significant difference in the C4AQ0 phenotype between the SLE and control groups existed in the absence of TNFa2, all individuals carrying a TNFa2 allele (whether C4AQ0 or not) were excluded from the analysis. These represented TNFa2 negative individuals (Table 4.16). The increase in C4AQ0 in the patient group was still significant (p=0.005).

Likewise, to examine if a significant difference in TNFa2 is still apparent between the two groups in the absence of C4AQ0, all individuals with C4AQ0 (irregardless of TNFa type) were excluded from the analysis (Table 4.16). Of 24 C4AQ0 negative individuals in the SLE group, 50 % had at least one TNFa2. In the normal Korean group, only 16% of 36 C4AQ0 negative individuals had TNFa2. The difference was

Table 4.16: Contingency tables and probability values for phenotypic comparison of C4AQ0 and TNFa2 between Korean SLE and Korean normals

all individuals with C4 types

	patient	normal
C4AQ0 positive	19	10
C4AQ0 negative	24	36

p=0.041

all individuals with TNFa types

	patient	normal
TNFa2 positive	20	10
TFNa2 negative	28	40

p=0.028

TNFa2 negative individuals

	patient	normal
C4AQ0 positive	13	6
C4AQ0 negative	12	31

p=0.005

C4AQ0 negative individuals

	patient	normal
TNFa2 positive	12	5
TNFa2 negative	12	31

p=0.004

significant (p=0.004).

The *allelic* frequency of TNFa2 in the individuals not carrying a C4A null gene (i.e. were C4AQ0 negative) in the Korean SLE and normal groups was also examined. There was a significant increase (p=0.007) of TNFa2 alleles in the Korean SLE compared to normals (12/48 to 5/72 respectively).

In summary, there are increased frequencies of C4AQ0 and TNFa2 in the Korean SLE group compared to the Korean normal group, and their increases are independent of each other.

CHAPTER 5: DISCUSSION AND CONCLUSIONS

5.1 Establishment of TNFa Typing

The TNFa microsatellite is a dinucleotide repeat found in the central region of the MHC on chromosome six (Nedospasov et al. 1991). As has been shown for many other dinucleotide repeats (Litt and Luty, 1989; Weissenbach et al., 1992), this CA repeat has been demonstrated to vary in the number of its repeats between different individuals (Nedospasov et al., 1991; Jongeneel et al., 1991). Because the MHC is a region of great interest in the study of immunology and disease, this microsatellite offered another locus that, although having no known function itself, may serve as a useful polymorphic marker for genes in the region of its occurrence.

The initial part of this study was concerned with the reliability of the TNFa typing methodology for use in this laboratory, not only as part of this project but possibly in future research here as well. There were two primary concerns addressed in this regard. These were

1) the naming of alleles consistent with the literature and
2) consistent and accurate typing of samples.

5.1.1 Naming of Alleles

Alleles were designated by number, one representing the smallest allele. The same nomenclature as published in the definitive papers on this microsatellite (Nedospasov et al., 1991; Jongeneel et al., 1991) was used in this study and special care had to be taken to assess that alleles were appropriately assigned. Because the length of the different dinucleotide repeat variants differ by only two base pairs (Table 4.1), it is important not to confuse alleles with closely related sizes. Sizes of alleles from DNA from several individuals, some of which later served as the control panel for other gels, were determined using sequenced M13mp18 DNA. To determine if assigned alleles were consistent with published nomenclature, seven of the homozygous workshop cell lines from the 10th International Histocompatibility Conference that had been previously typed by Jongeneel and colleagues (1991) were typed and the results compared (Table 4.2). All seven agreed perfectly. This allowed the results to be compared with confidence to that of other papers using this same microsatellite. Fortunately, the two alleles that flank the most common allele were both uncommon alleles and this provided further confidence in the naming of the alleles.

5.1.2 Consistency of TNFa Typing

To assist with determining consistency of the TNFa typing, 73 samples were repeated on various gels and a large extended family was examined for haplotype inconsistencies.

The difficulty in typing microsatellites is that shadow bands often occur (Litt and Luty, 1989). Usually these are fainter than the major band and occur in a ladder effect decreasing in size by the length of the repeat; in the case of a dinucleotide repeat by two base pairs. The greatest difficulty with the shadow bands occurs when they overlap a smaller allele, especially if the difference in the two alleles is only one repeat unit (that is, the person is heterozygous for consecutive alleles). Therefore, it would be expected that this situation would have the highest frequency of errors in typing. This is indeed what was observed in both the retyping data and the family study. For example, of 73 people retyped, the only ambiguity was in determining homozygosity. In either the first or repeated typing, 3 individuals were classified as homozygous in one typing and questionable homozygous in the other typing. In the family study, the only two individuals that were mistyped (Table 4.4) belonged to the questionable heterozygote category. They had initially been typed as most likely heterozygous for consecutive alleles but analysis of family data followed by

reexamination of the autoradiographs indicated that they were actually homozygous. In the family data the error lay in reading the autoradiographs. In the retyping of the samples mentioned previously the differences reflect some ambiguity in reading of autoradiographs but also some small differences in the banding patterns between different gels.

However, overall, the naming and typing of alleles were accurate and consistent, partly as a reflection of using good internal controls. The DNA panels indicated in Table 4.3 allowed for much easier assessment of alleles and comparison between gels. Additionally, the amplification and running of a sample with consecutive TNFa alleles on every gel helped in distinguishing heterozygotes with consecutive alleles from homozygosity for the larger allele. For future work, it would be beneficial to have all autoradiographs independently scored by two different people and the results compared. In this way errors in reading of the autoradiographs, such as the three homozygous individuals typed incorrectly as heterozygous for consecutive alleles, should be detected.

Technology for microsatellite typing has progressed from its earlier days. As indicated in the Materials and Methods section, the size of the PCR-amplified region containing the TNFa microsatellite was identified by radioactively labelling the DNA strand, separating the fragments on denaturing

acrylamide gels and visualizing the fragments through autoradiography. This procedure for microsatellite typing is still used by many labs, and indeed, is still probably the method most in use. However, it is not the only option. Work in typing microsatellites using silver staining for visualization instead of radioactivity has proven successful, especially with repeats larger than dinucleotide repeats (Santos et al., 1993). This method offers the considerable benefit of not using radioactivity and has been reported to be less affected by the `stutter bands' occurring in a ladder effect behind the major bands (Luqmani et al, 1997). However, it does require more manipulation after the gel has been run for the purposes of staining and destaining. A more popular method for large labs with access to the appropriate equipment is fluorescence-based DNA sizing of microsatellites using automated DNA sequencers and fluorescent labelled primers. This has been successfully used with a variety of different microsatellites including the dinucleotide repeats in the TNF region (Hajeer et al., 1996). In this situation, instead of being radioactively labelled, primers are labelled with fluorescent dyes. Once the PCR is completed, the samples are run on denaturing sequencing gels and a scanner `reads' the gel for the appropriate fluorescence and then offers a visual picture on a computer screen. Besides not using

radioactivity, this method offers some degree of automation especially in the reading of the gel. Because different primers can be labelled with different fluorescent dyes, samples can be multiplexed and run on the same gel. In standard assays using radioactivity, this can only be done if the different microsatellites vary significantly in length and none of the alleles overlap in size. However, the use of fluorescently labelled primers and automated sequencers requires the use of expensive technology, and, thus, many labs still rely on the primary technique of labelling samples radioactively. If conditions are carefully optimized and proper controls are in place, all the methods should give accurate and consistent results such as those obtained for this project.

5.2 Allele and Haplotype Associations

Because many of the alleles of the five loci did not occur frequently, it was difficult to determine if associations between TNFa variants and HLA-B alleles or TNFa variants and complotypes existed. Of those occurring more often in this population, many of the TNFa variants occurred with a variety of HLA-B alleles and a variety of complotypes. However, all 19 chromosomes with HLA-B8 also carried TNFa2 (the most common TNFa allele) and 19 of the 22 HLA-B7

haplotypes occurred with TNFa11. For complotypes, BfS;C4AQ0;C4B1 occurred 17 out of 19 times with TNFa2. This is also reflected in the extended haplotypes where HLA-B8;BfS;C4AQ0;C4B1 occurs with TNFa2 15 times.

Certain alleles have been found to occur together more frequently than expected by their individual frequencies in the population. Therefore, similar MHC haplotypes were often seen in unrelated individuals in the population. The nature of this association has been investigated by several researchers. DNA sequences between these polymorphic loci, in coding and noncoding regions, have also been demonstrated to be identical between these people (Wu et al., 1992; Pei et al., 1991; Abraham et al., 1992). Thus, it has been proposed that these combinations represent ancestral haplotypes that have been conserved *en bloc* from a very remote ancestor (Degli-Esposti et al., 1992a). Work by Degli-Esposti and colleagues (1992a) has demonstrated that whole haplotypes are more common than fragments in the population that they studied. This was also demonstrated in this study where there were 42 HLA-B;TNFa;Bf;C4A;C4B complete ancestral haplotypes (no DR information was available for this study) compared to 24 haplotypes containing only fragments of these delineated haplotypes. The suggested explanations for maintenance of these haplotypes include a)selection favouring this particular

combination of alleles in the regulation of the immune response (Bodmer and Bodmer, 1978; Wu et al., 1992) and b) inhibition of recombination or preferential recombination with homologous regions thus reconstituting the ancestral haplotype (Degli-Esposti et al., 1992a; Wu et al., 1992). There is evidence to suggest that recombination points are not randomly distributed over the MHC. Data from Abraham and colleagues (1993c) suggest that the recombination rate varies over the MHC but is generally low. They suggest that recombination within certain sections of the MHC is inhibited by the high degree of polymorphism in coding and noncoding regions within these blocks.

It is important to characterize these haplotypes. They may aid in determining genetic similarity between different populations which can be useful in epidemiology and understanding the evolution and migration of the human species. Many autoimmune diseases have complex associations with various alleles or combinations of alleles at several MHC loci. Often autoimmune diseases are associated with ancestral haplotypes and therefore with alleles at multiple loci (Degi-Esposti et al., 1992). It can be difficult to determine which of these genes are the loci directly involved in disease susceptibility and which are merely markers for other linked loci in the region. However, characterization of the DNA

content of ancestral haplotypes and identification of recombinants can help by delineating the shared region of the chromosome in the affected people, in much the same way as recombinant haplotypes narrow the region of interest in linkage studies of families. Therefore, the greater the number of polymorphic markers identified and characterized in ancestral haplotypes, the better the chances of narrowing the region involved. Indeed, work on mapping the susceptibility gene(s) for myasthenia gravis has been carried out using this technique (Degli-Esposti et al., 1992b).

There is a high frequency of the extended HLA-B8, BfS, C4AQ0B1 haplotype in this Newfoundland population as demonstrated in Table 4.7. This 8.1 ancestral or extended haplotype occurred 15 times out of the 75 independent haplotypes (20%). These haplotypes were considered independent based on the family interviews, available records and resulting pedigree analysis. There is always the possibility that the individuals are related more distantly than available data indicated. Nevertheless, this haplotype has been reported to occur in approximately 12% of an Australian population studied by Degli-Esposti and colleagues (1992a). In the Newfoundland population all of the 8.1 haplotypes occurred with the TNFa2 allele which supports other reports in recent years which have also shown this association

in the populations they have studied (Degli-Esposti et al., 1995; Garcia-Merino et al., 1996).

In looking at the whole haplotype comprising HLA-B, Bf, C4A, C4B and TNFa, there were some cases where independent haplotypes had the same HLA-B, Bf, C4A and C4B alleles but not the same TNFa variants, even though the TNFa locus occurs between HLA-B and the complotype loci. There were five examples where at least 2 independent haplotypes were identical for all alleles except the TNFa microsatellite. Two of these have the S31 complotype which is the most common complotype (Alper et al., 1983). Because DR data were not available, it is difficult to ascertain whether one or both of the haplotypes is a recombinant with another ancestral haplotype also bearing the common S31 complotype. In the other 3 cases, the difference in the TNFa allele may represent multiple historic recombinations or the greater instability of the microsatellite compared to other loci. This would lead to one allele being converted to another. Other authors (Abraham et al., 1993b) have also shown that the same ancestral haplotype may have a different TNFa allele and hypothesized that in vivo "slippage" may account for alleles that only differ by a single repeat unit. Slipped-strand mispairing has been proposed as one of the mechanisms in generating simple repetitive DNA sequences. Repetitive DNA consists of a core

unit, for example CA, that is repeated a number of times in an individual's DNA. In essence, one CA in the strand can mispair with the neighbouring complementary pair on the other strand. This can, when followed by DNA replication or repair, lead to an insertion or deletion of one, or possibly more, repeat units (reviewed by Levinson and Gutman, 1987). There were two haplotypes where this would seem a definite possibility: HLA-B12;BfS;C4A3;C4BQ0 (TNFa6/4) and HLA-B35;BfF,C4A3;C4BQ0 (TNFa4/5).

Microsatellites do have a higher rate of mutation than most loci (Jorde et al., 1998) with some approaching 10^{-3} per generation. Therefore, it is not surprising that some haplotypes may share alleles at flanking loci but differ in their TNFa variants.

Nevertheless, it would appear that the TNFa microsatellite is considerably stable. This is reflected in the associations of various extended haplotypes outlined in Table 4.7 with TNFa variants. Linkage disequilibrium between various TNFa variants and specific alleles or haplotypes has been demonstrated by a number of researchers including Jongeneel and colleagues (1991), Sturfelt and coworkers (1996), Garcia-Merino et al. (1996) and Turbay and colleagues (1997). Similar to this study, strong association of TNFa2 with HLA-B8;BfS;C4AQ0;C4B1 was identified as well as HLA-

B7;BfS;C4A3;C4B1 with TNFa11. However, because we do not have DR data for our family study from which the haplotypes were derived, it is difficult to determine those extended haplotypes that may be unique to this population and those haplotypes that are recombinants of two ancestral haplotypes with the same complotype. HLA-DR typing of this family and combination of DR data with already existing information on the extended haplotypes would make an interesting future project. It would also be interesting to try and determine if the three cases in which two haplotypes shared alleles at all loci studied except for TNFa are possible multiple recombinants. This would require much more extensive work in characterizing the alleles of all nearby genes in the region around the microsatellite and possibly sequencing as well. If these haplotypes represent the same extended haplotype but with a mutated microsatellite, it would be expected that the surrounding genes and DNA would be identical. If multiple recombinations have occurred, there is a possibility that other polymorphic loci in the area will have different alleles as well.

Future studies could also include typing of the other microsatellite loci found in the TNF region. This would provide more information to include in the haplotype analysis. Although this thesis represents the conclusion of this

project, collection of data for this project started in 1992 and continued into 1994. Thus, at the time the information was accumulated, less information about microsatellites in the TNF region was known than currently. The initial papers that described the TNF microsatellites (Nedospasov et al., 1991 and Jongeneel et al., 1991) reported the existence of four microsatellites in this region, labelled TNFa, TNFb, TNFc, and TNFd. Information was only provided on the first three, and, of these, the most polymorphic was TNFa, approximately 3.5 kb away from the TNF-β gene. Twelve alleles had at that point been described. TNFd was estimated to be about 10 kb downstream of the TNF loci and was not well characterized in these papers. TNFc was within the first intron of TNF-β but was only reported to be biallelic. Although TNFb was also quite polymorphic with six alleles reported, it was located next to the TNFa microsatellite, approximately 20 base pairs away. Because of its greater polymorphism, TNFa was chosen for this study, and because of the tight physical linkage of TNFb, there seemed little extra information to be gained from doing this microsatellite. However, more recently, there has been evidence to suggest that although TNFb is in close physical linkage with TNFa it may have evolved independently and may help identify different haplotypes. Garcia-Merino and colleagues (1996) identified 30 different TNFabc haplotypes

among 80 normal haplotypes, indicating that the addition of the TNFb and TNFc microsatellites does offer greater ability to discriminate between haplotypes that carry the same TNFa allele. Weissensteiner and Lanchbury (1997) hypothesized that despite the number of different haplotypes generated using the TNF microsatellites, the haplotypes could be categorized into one of three different lineages. Although it is advantageous to examine all the microsatellites in the TNF region, TNFa still remains the most polymorphic and thus most likely to offer the most information in haplotype and disease studies.

5.3 TNFa Alleles and Null Alleles of C4

One of the objectives of this study was to assess whether particular microsatellite variants were markers for the null alleles of the fourth component of complement. Null alleles are those from which no protein is produced. Null alleles can only be definitely assigned through family studies or by DNA characterization. In the haplotypes defined in the large kinship, null alleles were assigned with certainty based on the identification of a family member who was homozygous for either C4AQ0 or C4BQ0 alleles and, thus, did not produce the protein of that isotype. However, null alleles are often assigned in the literature by the use of densitometry. Null alleles are assigned based on relative staining of C4A to C4B

bands separated on electrophoretic typing gels. There can be considerable overlap in protein produced from those with a null and those without a null. If a particular microsatellite variant was associated with either the C4A or C4B null alleles, this, in conjunction with densitometry, would allow greater confidence in assigning null alleles in the absence of family studies, in much the same way as having HLA-B8 and DR3 together in a person suggests the presence of the deleted C4A gene. However, no particular allele was solely associated with either C4AQ0 or C4BQ0 in this study.

In the large kinship, definite nulls could be assigned. Although TNFa2 was associated with the BfS;C4AQ0;C4B1 complotype in this group, it was also seen with several other C4A alleles including C4A2,C4A3 and C4A4 and is the most common variant in this population. Also, the BfS;C4AQ0;C4B1 complotype occurred in most examples with HLA-B8 and most likely represents the 8.1 ancestral haplotype which is known to carry a deleted C4A gene (Uring-Lambert et al., 1987). In total, there were five different haplotypes that carried a C4A null (Table 4.11). TNFa2 occurred in three but not in the other two. The C4BQ0 allele occurred on a greater number of different haplotypes and with a diverse selection of TNFa alleles (seven different TNFa alleles). Null alleles are defined on a phenotypic level by the lack of protein produced.

They do not necessarily represent a homogeneous population at the molecular level. Indeed it is known that some, such as the C4AQ0 associated with the 8.1 haplotype, are the result of deletions, while detectable deletions have not been identified in other nulls (Uring-Lambert, 1987). These results further support the existence of heterogeneity at the DNA level. The results suggest that these null alleles arose by different methods and would, therefore, not be expected to share a common TNFa allele.

The Korean population used for the SLE association study also provided an opportunity to examine the null alleles, defined in their population by densitometry. The nulls in the Korean group could not be assigned to TNFa alleles directly because no family study was undertaken. However, it was clear that no single TNFa allele occurred in all the individuals with C4AQ0 in either the normal or patient groups.

Again the fact that no association with a particular variant of TNFa could be seen is not surprising as these nulls may represent different mutations that resulted in loss of function. Restriction digests done by the investigators (Hong et al., 1994) indicated that none of these nulls represent the large deletion associated with the HLA-B8;BfS;C4AQ0;C4B1 haplotype, which is a rare haplotype in this population. Because the null alleles have not been assigned using family

studies, there is also the question of whether these are actually null alleles. Densitometry makes the assumption that the two genes for, and alleles of, the isotypes of C4 make equal amounts of protein if they have the same number of genes. Null alleles are defined by a band on an agarose gel of about half the intensity of the band(s) of the other isotype. However, Hammond and colleagues (1992) did show that there is considerable overlap in the amount of protein produced by individuals with different numbers of C4 genes. The C4AQ0 in the Korean population studied here actually represents low C4A protein relative to C4B protein. Although it is possible that the reason for this low total C4A is because of the presence of an allele that does not produce protein, there are other possibilities. For example, C4A and C4B, while having the same functions, are known to have different efficiencies in some of their functions (Schifferli et al., 1986; Paul et al., 1988; Kishore et al., 1988). This may lead to preferential depletion of one isotype over another in certain situations.

In conclusion, aside from the association between TNFa2 and C4AQ0 on the 8.1 ancestral haplotype, TNFa alleles provide no additional evidence to aid in assigning null alleles to either of the C4 isotypes. This most likely reflects the heterogeneity of the mutations at the DNA level that gave rise

to the similar null phenotypes.

5.4 SLE and the TNFa Microsatellite

The most frequent allele in the normal Newfoundland Caucasian population was TNFa2 but in the Korean population it was TNFa6. In the SLE populations from both regions, there was a significant increase in the TNFa2 microsatellite variant compared to the ethnically matched controls.

As reported in the previous sections, TNFa2 is associated with the ancestral haplotype 8.1 (HLA-B8;BfS;C4AQ0;C4B1;DR3) which occurs frequently in the Caucasian population (20% of the haplotypes in this population were HLA-B8;BfS;C4AQ0;C4B1). This haplotype is known to be increased in Caucasian SLE populations (Kemp et al., 1987). Thus, the increase in the TNFa2 variant in the Caucasian SLE could be explained by this. Indeed, of the 19 individuals with TNFa2 in the Caucasian SLE population, 13 occurred in people also carrying DR3. Although it was not possible to haplotype these people, the evidence in the literature and in this thesis suggests that there is a high probability of the two occurring together as part of the 8.1 extended haplotype. This makes it very difficult to identify which gene(s) are involved in susceptibility to SLE in this population.

In the healthy Korean population, HLA-B8 and DR3 alleles

are uncommon (Hong et al., 1994) and are not increased in the Korean SLE population nor is there an increase of the 8.1 haplotype. Indeed, no DR3 alleles occurred in any of the 50 normal Korean individuals and only two instances of DR3 alleles occurred in the 48 SLE patients that were a part of this study. In both cases where a DR3 occurred, a TNFa2 also occurred in the same person. However, 17 out of the 20 individuals with TNFa2 in the SLE population were not DR3 so the increase of the TNFa2 allele could not be attributed to an increase in DR3. Because TNFa2 occurred frequently with SLE but not DR3, this would seem to suggest that there is an independent risk factor in the central region of the MHC that is linked to the TNFa microsatellite.

There are many genes within this region of the MHC with immunological function and some with unknown function which could possibly be the disease susceptibility locus or loci. Certainly, the null alleles of the C4 loci are known to be associated with SLE which, given the importance of the protein products in inflammation and clearance of immune complexes, is not surprising. The frequency of the null alleles of the C4A isotype has been shown to be increased in SLE patients in a variety of different ethnic groups including Caucasian (Kemp et al., 1987; Partanen et al., 1988; Goldstein et al., 1988; Fronek et al., 1988) and Korean (Hong et al., 1994). However,

the nature of the null alleles differs in the two groups. As previously mentioned, in Caucasians it is usually associated with a 30 kb deletion encompassing the C4A locus and occurring as part of the HLA-B8, HLA-DR3 haplotype. The Korean SLE population used in this study has previously been reported to be also associated with an increased incidence of C4AQ0. In that study population, the null alleles were assigned by densitometry, and was not the deleted C4AQ0 common in Caucasian SLE (Hong et al., 1994). This, as discussed in Section 5.3, is probably more accurately described as low C4A protein relative to C4B protein. Nevertheless, the TNFa2 variant could be associated with these 'null' alleles but this does not seem likely. Analysis of the null allele in the Korean SLE, identified by densitometry, does not show an association with TNFa2, or any other TNFa allele, as discussed in Section 5.3. Only 4 out of 10 people with low C4A in the Korean normals and 6 out of 19 with low C4A in the Korean SLE patients had a TNFa2 allele. Therefore, even if these nulls are genuinely nonexpressed alleles, there is still no association between them and TNFa2, and thus would not be the cause of the increase in TNFa2 allele frequencies in the Korean SLE. Moreover, even if the C4AQ0 in the Caucasian and Korean populations are functionally the same, they are quite different at the DNA level. In the Caucasian population, it

represents a large deletion but in the Korean population it represents a present but possibly mutated, nonfunctional gene. Therefore, it would be surprising to find that they are both associated with the same TNFa allele, and, indeed, this is not the case.

Further analysis of the relative contributions of TNFa2 and C4AQ0 to SLE in the Koreans was examined in Section 4.4.3. The phenotypic frequency of both C4AQ0, as defined in this population, and TNFa2 was significantly increased in Korean SLE in the absence of the other. Allele frequencies of TNFa2 in individuals without C4AQ0 was increased in Korean SLE compared to controls ($p=0.0075$). This suggests that the increase in TNFa2 in Korean SLE does not reflect an increase in C4AQ0 and is a marker for something other than C4AQ0 or DR3.

It would appear that there may be two independent risk factors in the central region of the MHC, in the Korean SLE at least. One is the null allele of C4A, or a gene in linkage disequilibrium with it. The other is a gene or genes linked to the TNFa2 allele of the TNFa microsatellite. The tumour necrosis factor loci, 3.5 kb from the microsatellite, are possible candidates. As indicated in the introduction, (NZB X NZW)F1 hybrids have decreased production of tumour necrosis factor alpha (Jacob et al., 1991a) and this has been linked to

a microsatellite near the mouse TNF region (Jongeneel et. al, 1990). Moreover, treatment with recombinant TNF significantly delayed onset and severity of disease in these animals (Jacob et al., 1991a). Extrapolation of these results to the human condition has led researchers to demonstrate that patients with more severe SLE (lupus nephritis) have decreased TNF inducibility of cells *in vitro* and this is linked to the MHC (Jacob et al., 1990). It seems possible, therefore, that variation in the TNF loci is most likely to be quantitative instead of qualitative, i.e., it is the regulation of TNF that is most significant and not allelic forms of the protein (which seems to be very limited).

There have been a variety of papers that have investigated the relationship between polymorphisms within the TNF region and levels of production and inducibility of TNF-α and TNF-β. Some studies show associations while others do not. A study by Pociot and colleagues (1993b) showed a significant increase in TNF-α production with the TNFa2 allele and low production with TNFa6. However, because the study used heterozygous individuals, the effect of one allele may be masked or complemented by the other. Consequently, the results are complicated to interpret and compare. Moreover, different studies use different inducing agents, different concentrations of inducers, different cell populations, and

different time frames. Thus, comparison of results from different investigators is difficult as is extrapolation to the *in vivo* situation.

Three other studies in recent years have investigated the TNFa microsatellite in SLE. D'Alfonso and colleagues (1996) found no association with any of the TNFa alleles in the Italian population they studied. Sturfelt and colleagues (1996) and Hajeer and colleagues (1997) reported an increase in the TNFa2 allele in their Caucasian SLE populations but it occurred as part of extended MHC haplotypes.

This is the first study to investigate the TNFa allele in the Korean SLE population. SLE is a complex, multifactorial disease with diverse clinical manifestations. A combination of defects most probably has to develop for SLE to occur and the actual combination may be different in different individuals (Stone, 1988). The variability of the SLE phenotype may be a reflection of these different combinations of both genetic and environmental defects. The more similar the phenotype, the greater the likelihood of detecting gene association with that phenotype of the disease. Hajeer and colleagues (1997) demonstrated increased allele frequency of TNFa2 in patients who possessed anti-la and/or anti-ro autoantibodies. Clinical features in the Korean SLE patients in this study may be more homogenous and thus more likely to

show an association. The clinical data was not available at this time, but it would certainly be worthwhile to investigate. It would also be interesting to examine if C4AQ0 Korean SLE patients showed a different clinical phenotype than the TNFa2 patients. Of course, a larger sample set and other populations showing different MHC associations with SLE would be valuable in further TNFa microsatellite studies.

The increase of the TNFa2 allele in this SLE population combined with TNF protein and association studies in the literature suggests a potential role for regulatory variation of the TNF loci in susceptibility to SLE. Further studies using the Korean subjects involving the production and inducibility of tumour necrosis factor alpha and beta would be interesting to investigate in

a) normals with TNFa2 compared to other TNFa alleles and

b) SLE patients with TNFa2 compared to other SLE patients with different TNFa alleles.

Certainly, the association of TNFa2 with SLE in two different ethnic groups, and the association of TNFa2 with SLE in a Korean population which does not show the extended HLA-B8 haplotype and C4AQ0 deletion seen in Caucasians, is an important observation worthy of further investigation.

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