CHAPTER 1

INTRODUCTION

Background and Rationale

Graves' disease (GD) is a typical organ-specific autoimmune disease that affects people all over the world. The condition involves a complex interaction of multiple genetic, environmental and endogenous factors. Several factors can influence susceptibility to Graves' disease, the more important of which are hormone level, iodine deficiency, stress and smoking. Genetic factors are important predisposing factors, but not determinative. GD, characterized by thyroid gland hyperfunction, is caused by the production of thyroid-stimulating immunoglobulin(TSI) against the thyroid-stimulating hormone receptor (TSH-R) on thyroid cells, leading to hyperthyroidism. (Feliciano, 1992). Although the cause of GD is unknown, the role of helper T lymphocytes in activating antibody-producing B cells is established.

GD is mostly found simultaneously with other autoimmune diseases such as Type I diabetes mellitus (IDDM), myasthenia gravis and rheumatoid arthritis (RA) within the same individual or family, and predominately in females with the highest-risk ages between 40 and 60 years (Weetman, 1994). White and Asian populations are at higher risk than black groups.

Associations between several autoimmune diseases and antigens of the major histocompatibility complex (MHC) have long been recognized. HLA-DQ and HLA-DR are highly polymorphic and exhibit strong linkage disequilibrium (Bidwell, 1988). The complexity has been further intensified by developments in HLA typing technology such as polymerase chain reaction and sequencing technique. Studies in different ethnic populations have led to the observation that HLA- DRB1*03, DRB1*08 , DRB3*0101, DRB3*0202 and DQA1*0501 are associated with the disease in Caucasians and DRB1*0701 is protective. (Farid et al., 1979, Yanagawa et al. 1994, Chen et al., 1999) It has been reported that different HLA associations are found in Graves' disease in Orientals, although the number of studies is rather small. For instance, HLA-B46, DR9, and DQB1*0303 were associated with GD in Hong Kong Chinese. (Cavan et al., 1994 and Wong et al., 1999) In Taiwan, HLA-A*0207, B*2704, B*4601, and DRB1*0901 have been reported. (Huang et al., 2003) HLA-A2, B46, DRB1*0803, DRB1*1403, and DPB1*0501 were found in

positive associations with GD in Japan. (Katsuren E.et.al. 1994) However, because of strong linkage disequilibria within this region, the primary etiological variant(s) remains unknown. Study on HLA and disease association lead to characterize the causative HLA factor authentically responsible for the association and probably understand the initiating mechanisms or the promoting of the disease progression.

Literature Review

A. Major Histocompatibility Complex

The major MHC in humans, also known as the human leukocyte antigen (HLA) in humans, is a large genomic region of highly polymorphic genes. The HLA region covers of about 3.6 Mbp depending on the haplotype, spans on chromosome 6p21.1 to p21.3, with class II, class III and class I genes located from the centromeric to the telomeric end. This set of genes is known to play a key role in the immune system, autoimmunity, and reproductive success. The HLA region can be split into three different parts (from centromere to telomere) as first proposed by Jan Klein in 1977: class II (850 kb), class III (1100 kb), and class I (1800 kb) In addition, a class IV region located at the telomeric end of the class III region has also been proposed. (Gruen and Weissman, 1997)

The HLA class I and HLA class II regions contain genes that encode cell surface glycoproteins. One of the major roles of their products in the physiological immune response involves the antigen presentation to T cells. The class I region encodes HLA-A, -B and -C molecules, expressed on the cell surface of nucleated cells and involved in the presentation of peptides predominantly derived from intracellular proteins to CD8+ cytotoxic T cells (CTLs). The class II region encodes many membrane-bound proteins expressed on the cell surface of B lymphocytes, macrophages, monocytes, dendritic cells and endothelial cells, that interact exclusively with CD4+ helper T cells (THs). The segment of DNA in between these two regions, generally termed as the class III region, contains many genes encoding proteins of diverse structure and function such as tumour necrosis factor (TNF), heat shock proteins (Hsps) and complement proteins (C2, C4). (Powis et al., 1995). The HLA genes are expressed co-dominantly. Each individual has two variant forms of the product of a particular locus. The set of HLA loci that are inherited together on a single chromosome is termed the haplotype.

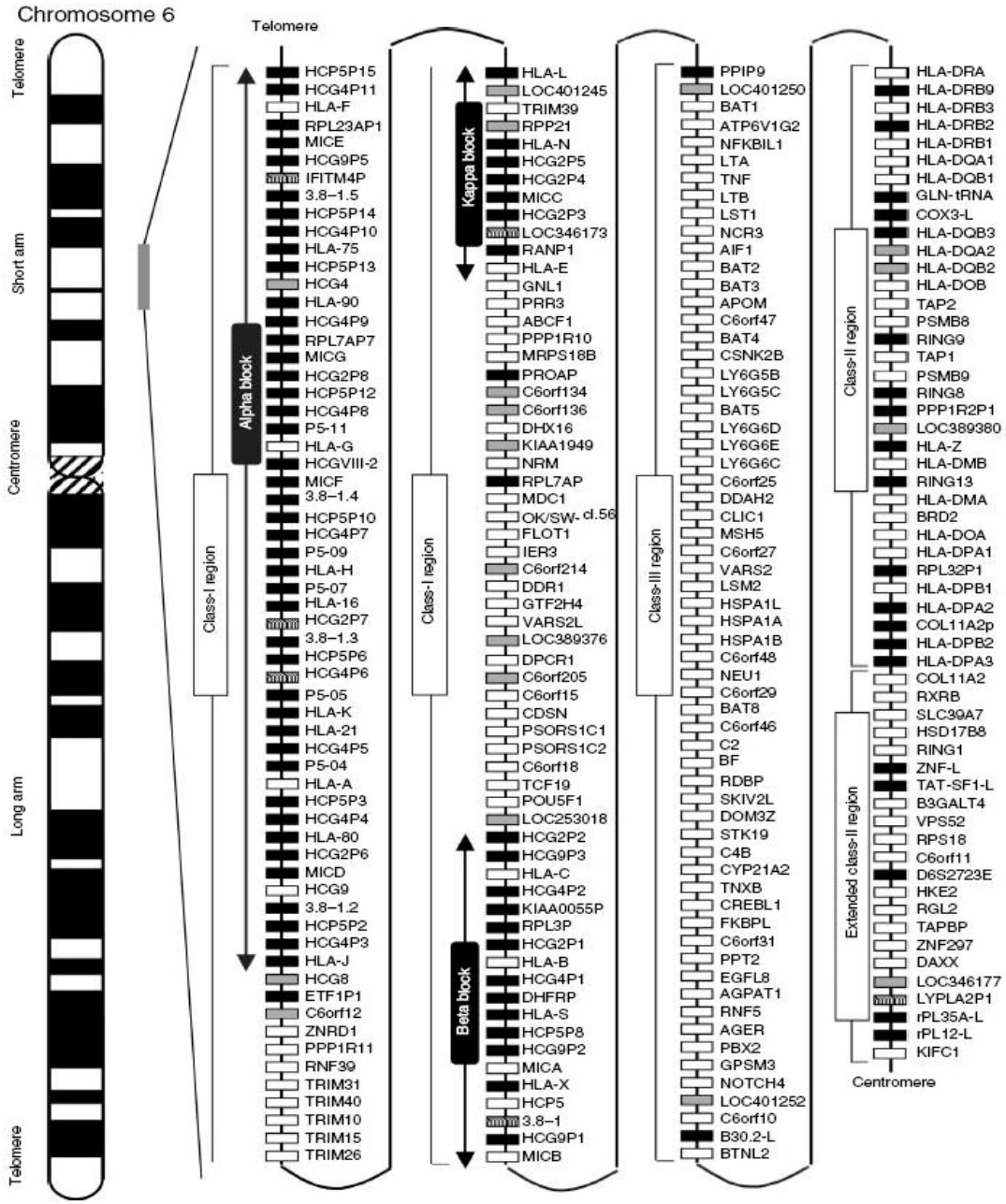

Figure 1 **Gene map of the HLA region.** The regions separated by arrows show the HLA sub-regions, such as classical class-I, class-III, classical class-II and extended class-II regions from telomere (left and top side) to centromere (right and bottom side). White, grey, striped and black boxes show expressed genes, gene candidates, non-coding genes and pseudogenes, respectively. The location of the alpha, beta and kappa blocks containing the cluster of duplicated HLA class-I genes in the class-I region has been indicated. (Shiina et al.,2004)

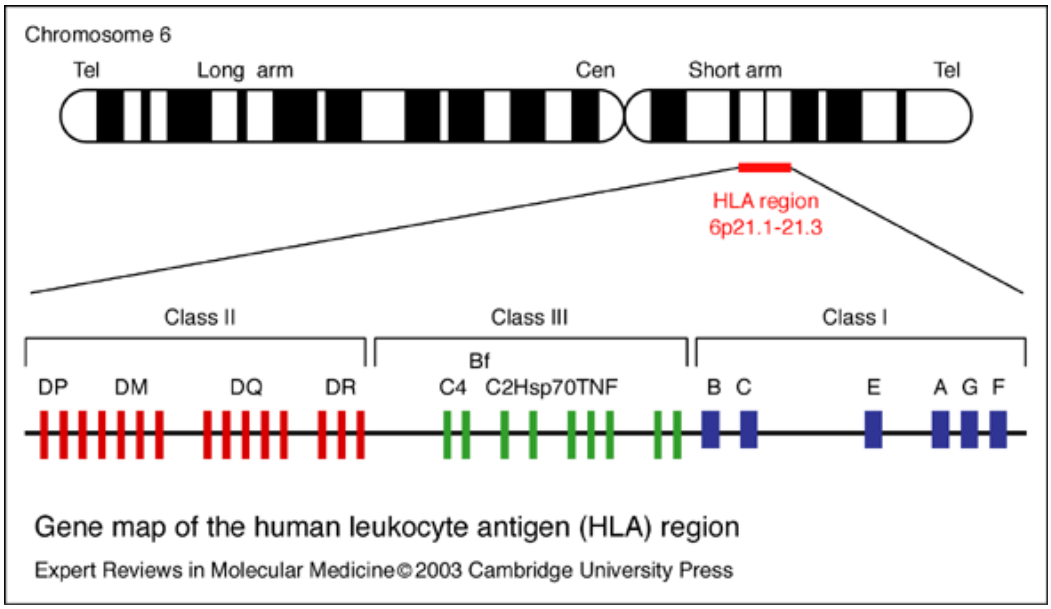

Figure 2 Gene map of the human leukocyte antigen (HLA) region. The HLA region spans $4x10^6$ nucleotides on chromosome 6p21.1 to p21.3, with class II, class III and class I genes located from the centromeric (Cen) to the telomeric (Tel) end. HLA class I molecules restrict CD8+ cytotoxic T lymphocyte function and mediate immune responses against 'endogenous' antigens and virally infected targets, whereas HLA class II molecules are involved in the presentation of 'exogenous' antigens to T helper cells. The HLA class III region contains many genes encoding proteins that are unrelated to cell-mediated immunity but that nevertheless modulate or regulate immune responses in some way, including tumour necrosis factor (TNF), heat shock proteins (Hsps) and complement proteins (C2,C4)(expert reviews in molecular medicine Vol. 5; 24 January ; 2003 Cambridge University Press)

A. 1 Genomic organization of the HLA genes

A. 1.1 The HLA class I gene

The class I gene at the telomeric end, spread over a region of 1800 kb, contains the six expressed HLA class I genes: the three classical (HLA-A, HLA-B, and HLA-C), the three

non-classical (HLA-E, HLA-F, and HLA-G); the two class I chain-related (MICA and MICB) genes and 12 non-coding genes or pseudogenes (HLA-S/17, HLA-X, HLA-N/30, HLA-L/92, HLA-J/59, HLA-80, HLA-21, HLA-K/70, HLA-16, HLA-H/54, HLA-90 and HLA-75). (Marsh et al, 2005) The heavy (α) chain of the class I molecules form heterodimers with the non-MHC gene encoded b2-microglobulin, the invariant light chain of HLA class I molecules. The α gene is comprised of eight exons. (Hansen et al., 1993) The first exon is short and encodes a signal peptide which directs the insertion of the MHC I molecule into the endoplasmic reticulum during translation. Exons two through four are all similar in size and encode the three extracellular domains of the protein (α1, α2 and α3). The α1 and α2 domains, which form the walls of the cleft where peptide binding occurs, are polymorphic. The fifth exon encodes the transmembrane domain, while exons six, seven and eight encode cytoplasmic sections. The introns vary in length from 33 to 599 base pairs. The gene for β2 microglobulin encoding is on chromosome 15. It consists of 4 exons but most of the coding region is found in the second exon (Austyn and Wood, 1993).

HLA-A and HLA-B were first characterized by serological techniques, likewise the HLA-C. The serological specificity of HLA-A and HLA-B are highly polymorphic whereas that of HLA-C is less polymorphic. The recognized serological and cellular HLA-A,-B and -C specificities are 28, 60 and 10. (McClosky et al., 1992). Based on molecular techniques, HLA Class I alleles assigned as of December 2004 can be classified to 349 allelic forms of HLA-A , 627 of HLA-B and 182 of HLA-C.(Marsh et al, 2005)

The 2004 Nomenclature Committee recognized more HLA class I genes and pseudogenes, all in the class I region: HLA-E, -F, -G, -H, -J, -K, -L, -N, -P, -S, -T, -U, -V, -W, -X, -Y and HLA-Z (a Class I gene fragment located within the HLA Class II region). Among these, only HLA-E, -F and -G are expressed. Some are defined as pseudogenes and their functions are still unclear, while some encode gene fragments associated with β2 microglobulin. (Marsh et al, 2005)

A. 1.2 The HLA class II gene

The HLA class II region spans approximately 850 kb at the centromeric end of the class I domain. There are three main subregions in the order centromere -DP-DQ-DR-telomere. The other class II genes, HLA-DZ and HLA-DO, lie between the HLA-DP and HLA-DQ subregions. The DR and DQ genes encode the α and β peptide chains of the HLA class II molecules, involved in processing antigens for presentation. The DM and DO genes are involved in the recruitment of antigens by class II molecules. The DP genes, DPA1 and DPB1, code for the α and β peptide chains that constitute class II molecules and, similar to DR and DQ, are also involved in antigen presentation to the CD4+Th cells. The HLA class II genes each contain five or six exons. The first exon of the class II α and β genes encodes the hydrophobic leader peptide, the second and third exons encode the two extracellular domains, α1α2 and β1β2 domains, which are very polymorphic, especially the α1 and β1 regions, the fourth exon encodes the transmembrane cytoplasmic domain and part of the 3' untranslated (3'UT) region of the mRNA, the fifth exon encodes the rest of the untranslated region and the last exon 6 encodes the remaining cytoplasmic portion and 3' UT region (Hansen et al., 1993).

The HLA-DR subregion contains genes coding for the alpha (the DRA gene) and beta (the DRB gene) chains. There is only one α gene or A gene (HLA-DRA) and nine β genes (HLA-DRBl through DRB9) consisting of 4 expressed genes, DRB1, DRB3, DRB4 and DRB5, and 5 pseudogenes, DRB2 and DRB6-9. Within the DR molecule the beta chain contains all the polymorphisms specifying the peptide binding specificities. Allelic variants of DRB1 are linked with either none or one of the genes DRB3, DRB4 and DRB5. (Anderson et al., 1994). Not all the HLA-DRB genes are present in a single haplotype, but are divided into 5 different groups of DR haplotypes in humans: DR1 (major haplotypes DRl and DRlO), containing DRBl and DRB6 loci; DR51 (haplotypes DRl5 and DR16) consisting of DRBl, DRB6 and DRB5; DR52 (haplotypes DR3 and DR11-DR14) bearing DRBl, DRB3 and one pseudogene, DRB2; DR8 (haplotype DRB8) comprising of a single functional locus,DRBl; and DR53 (haplotypes DR4, DR7and DR9) containing DRBl and DRB4 genes and two pseudogenes, DRB7 and DRB8. All the haplotypes carry one additional pseudogene, DRB9 (Goncora et al. 1996). The genetic organization of HLA-class II region is shown in Figure 2.

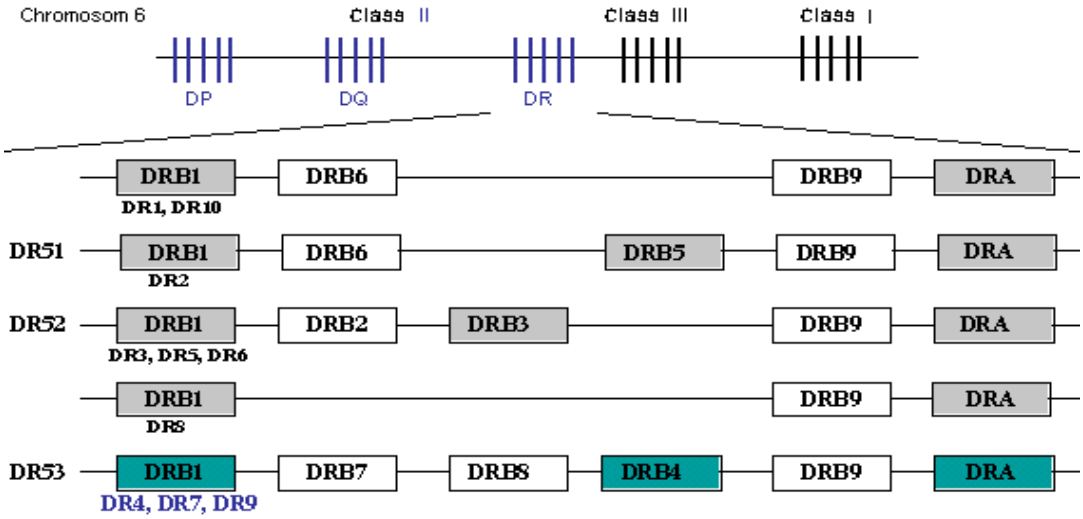

Figure 3 HLA-DR region mapping (Christian Heldt., 2004)

Based on standard molecular techniques, all recognized allelic forms of HLA-DRB genes assigned as of December 2004 can be classified into 394 alleles of the HLA-DRB1 locus, while the other three expressed HLA-DRB loci show less allelic polymorphisms; 41 of HLA-DRB3, 13 of HLA-DRB4 and 18 of HLA-DRB5. (Marsh et al, 2005)

The HLA-DQ and HLA-DP subregions comprise two α chain genes and two β genes (DQA1, DQB1, DQA2, DQB2, DPA1, DPB1, DPA2 and DPB2). The first gene of each pair, DQA1, DQB1, DPA1 and DPB1, is functional (Abbas et al., 1994). Both the α chains and the β chains of the HLA-DQ and HLA-DP proteins are polymorphic, and therefore each individual can express four different HLA-DQ and HLA-DP proteins. The polymorphisms of HLA-DQ assigned as of December 2004 by the WHO Nomenclature Committee represent 28 variants of HLA-DQA1, 61 of HLA-DQB1, 22 of HLA-DPA1, and 116 different expressed DPB1 alleles, excluding silent substitutions. (Marsh et al, 2005)

The non-classical MHC genes, HLA-DM and HLA-DO, act in the class II antigen-processing pathway. The HLA-DM eases peptide loading of class II molecules, whereas HLA-DO specifically suppresses its ability in peptide loading, resulting in a down modulation of the class II processing pathway. In addition, HLA-DM is a chaperone for MHC class II molecules and HLA-DO is a co-chaperone of HLA-DM. (Kropshofer et al., 1998) The numbers of

recognized alleles of HLA-DOA,-DOB,-DMA and DMB are 8, 9, 4 and 7, respectively. (Marsh et al, 2005)

A.1.3 The HLA class III gene

The HLA class III region, spanning approximately 700 kb, lies between the HLA-B locus at the telomeric end of 6p21.3 and the HLA-DR locus at its centromeric end. A class IV region has also been suggested, located at the telomeric end of the class III region. The class III region, which has been associated with susceptibility to numerous diseases, is the most gene-dense region of the human genome, containing 60-61 genes and approximately ~700 kb in size. There are an average of 8.5 genes per 100 kb with and an average intergenic distance of just under 3 kb. (Heiliget al.2003)

The class III region encodes for other immune components, such as complement components (e.g., C2, C4, factor B), heat shock proteins, cytokines (e.g., TNF-α) and some genes that are not involved in the immune system. Only a few of the genes in this region have a clearly defined and proven function. Among these genes, HSP70, TNF, C4A, C4B, C2, BF and CYP21 should be noted. The HSP70 genes, which show strong linkage disequilibrium (LD) with the HLA-DR alleles, encode cytosolic molecular chaperones and behave like a tumors rejection antigen by presenting the intracellular contents of cancer cells to the immune system. Because of their peptide binding and presenting abilities, it has been suggested that the HSP70 genes may be functional precursors of MHC molecules. (Srivastava and Heike, 1991) The tumor necrosis factor alpha (TNFA) and tumor necrosis factor beta (TNFB) genes encode cachectin and lymphotoxin-a molecules (LTA), respectively. (Webb and Chaplin, 1990) CYP21 (cytochrome P450, family 21) encodes 21-hydroxylase which is an enzyme critical in the biosynthesis of glucocorticoids, a deficiency of which can lead to the genetic disease congenital adrenal hyperplasia.(Levine et al, 1978) C2, C4A and C4B encode some of the complement proteins that help clear pathogens from an organism, while BF (complement component factor B) codes for factor B, which is also involved in immune response.(Campbell et al, 1986) An understanding of genetic variations within the class III region may be crucial in understanding some aspects of the genetic susceptibility to autoimmune disease.

A. 2 General features of the HLA class I, class II and antigen presenting pathway

A. 2.1 The HLA class I molecule and the endogenous pathway

The HLA class I molecules are membrane glycoproteins, synthesized in the rough endoplasmic reticulum (RER) and expressed on the surface of all nucleated cells (except foetal trophoblast cells) and platelets. They mediate immune responses against endogenous antigens, e.g. viral peptides or tumor antigens synthesized within the cytoplasm, to CD8+ cytotoxic T cells and activate them to kill cells. They are composed of 2 noncovalently associated polypeptides: an alpha chain (α-chain) and beta 2-microglobulin (β2M). (fig.3) The α-chain is the longer and heavier chain (MW 45 kD) (Hansen et al., 1993), consisting of 3 domains: α1(a membrane-distal domain), α2, and α3. The β2M, a polypeptide that is identical to a protein previously identified in human urine, called β2 microglobulin for its electrophoretic mobility (β2), size (micro) and solubility (globulin), is a light chain (MW 12 kD) that is encoded by a gene outside the HLA (chromosome 15 : q21-q22 segment) (Abbas et al., 1994)

The α 1 and α 2 are each composed of an alpha helix and form the sides of a peptide binding cleft, while the floor of the cleft is composed of eight anti-parallel β pleated sheets. (Brown et al., 1993). The amino acid side chains of either α helices or β strands are almost polymorphic (Austyn and Wood, 1993). The α3 domain (positions 223 to 229) is highly conserved among all class I molecules and is homologous to an Ig-like domain, and serves as a binding site for CD8 (Hansen et al., 1993). The α3 and β2-microglobulin domains interact with each other and anchor the alpha chains to the cell (Abbas et al., 1994). Within these domains, polymorphisms concentrate on three regions, called hypervariable regions (HVR): positions 62 to 83, 92 to 121, and 135 to 157. (Steinmetz and Hood, 1983) The antigenic peptides of 8–10 amino acids in size bind to the cleft with low specificity but high stability. (Rammensee et al, 1995) These peptides lie extended in the cleft by their main chain bonded to conserved residues of HLA class I, with a few interactions involving a side chain of bound peptides (Hansen et al., 1993). Interactions within the pockets might be direct hydrogen bonds between the side chain of the anchor residue and the class I residue or might be mediated by water (Matsumura et al., 1992).

The endogenous loading pathway begins when proteins in the cytosol are broken down into smaller peptides by a proteasome and transferred into the ER (endoplasmic reticulum) by the TAP1 and TAP2 complex (ATP-dependent transporting molecules that transfer peptides from the cytosol to the ER). Inside the ER, newly synthesized α-chains bind to a chaperone protein (calnexin). β2M then binds to the α-chain and calnexin is released, and the resulting complex binds with calreticulin (a chaperone protein), tapasin and Erp57. Tapasin and ERp57 form a stable disulfide-linked dimer within the MHC class I peptide-loading complex and bind the complex to TAP1 and TAP2. Then, a peptide is bound to the cleft of the MHC molecule, these three proteins are released and MHC is transported out of the ER to the cell membrane. Cytotoxic T cells will recognize this complex and initiate the appropriate immune response to kill this cell. (Monaco and Nandi, 1995)

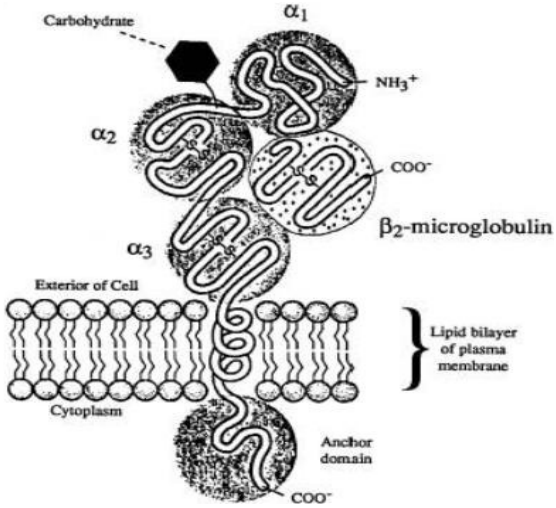

Figure 4 Structure and orientation of an MHC Class I Glycoprotein Molecule (Robert A. Freitas Jr., 1999)

A. 2.2 The HLA class II molecule and the exogenous pathway

The HLA class II molecules are cell-surface molecules found only on antigen presenting cells (APC), B lymphocytes, macrophages, monocytes, activated T cells, endothelial cells, Langerhans and related dendritic cells, that have similar structures to the HLA class I

molecules with subtle functional differences (Brown et al., 1993). The HLA class II mediates immune responses against exogenous antigens, composed of two noncovalently associated polypeptide chains, α (34 kD) and β (30 kD), both encoded by polymorphic MHC genes. Each chain can be divided into 2 extracellular regions, a transmembrane region and a cytoplasmic region (Abbas et al., 1994). The extracellular regions of the α and β chains have been subdivided into two domains, called α1 and α2, and β1 and β2, respectively. (Fig.4) Because the α and β chains are different, class II MHC molecules can be termed heterodimeric. Amino terminal α1 and β1 segments interact to form the antigen binding cleft (ABC), consequently, polymorphisms of class II molecules are concentrated in these domains encoded by the exon 2 of their class II A or B genes. Particularly in the DR locus, a greater degree of polymorphism is found in the β chain than in the α chain (Marsh and Bodmer, 1995). The floor of ABC formed by an 8 stranded antiparallel beta-sheet (4 strands contributed by each of α1 and β1) and the walls consist of two α-helices, allowing accommodation of larger peptides than in MHC class I (10 to 34 amino acids in length, with 12-16 optimal) (Chicz et al., 1993). Hypervariable regions tend to be found in the walls of the cleft. α2 and β2 segments are folded into nonpolymorphic Ig domains. They are members of the Ig superfamily, like the class I α3 and β2 microglobulin regions. The residues 241 to 255 in the β2 domain are the major CD4+ binding site (Konig et al, 1986), while polymorphic residues between positions 180 and 189 determine the quality of CD4+ interactions. (Fleury et al., 1995) The transmembrane regions expand from the α2 and β2 domains in the carboxy terminus and contain approximately 25 residue hydrophobic transmembrane stretch. This region is followed by a short hydrophilic cytoplasmic segment whose function is little understood (Abbas et al., 1994). The exogenous loading pathway begins by phagocytosis of extracellular proteins caused by, for instance, a foreign agent, an organism, bacteria, etc., lysed protein then leaches into vesicular compartments by APCs. Then, the lysosome will fuse with the phagosome to become a phagolysosome and the antigen will be degraded into smaller peptides. Both chains of the class II molecules are synthesized in the ribosomes which are associated with the RER, and then transported into endosomes. The folded and assembled chains migrate to the phagolysosome with the assistance of a chaperone molecule, such as calnexin. The invariant chain (Ii or CD74) assists in the folding and assembly of α and β chains by blocking the peptide binding site

temporarily to prevent binding to peptides in ER. (Sant and Miller, 1994) Class II-associated invariant chain peptides (CLIP) are then transported to the MHC class II compartment (MIIC; endosomal vesicles formed by fusion of exocytic vesicles from ER and endocytic vesicles). The endosomal/lysosomal system facilitates removal of CLIP by acidic conditions, and the internalized processed peptide with the appropriate sequence motif binds to the class II molecule. (Harding CV., 1996) HLA-DM, a non-polymorphic MHC-like molecule as a dedicated chaperone in MIIC, prevents the functional inactivation and aggregation of empty HLA-DR $\alpha\beta$ dimmers. (Sloan et al., 1995) The DR-peptide complex migrates to the membrane to present the antigen to the CD4+ T-helper cells. Only properly loaded MHC II molecules will be sufficiently stable to reach the surface. Bound peptides are stabilized in the groove by a hydrogen bond between the HLA molecule and peptide backbone.

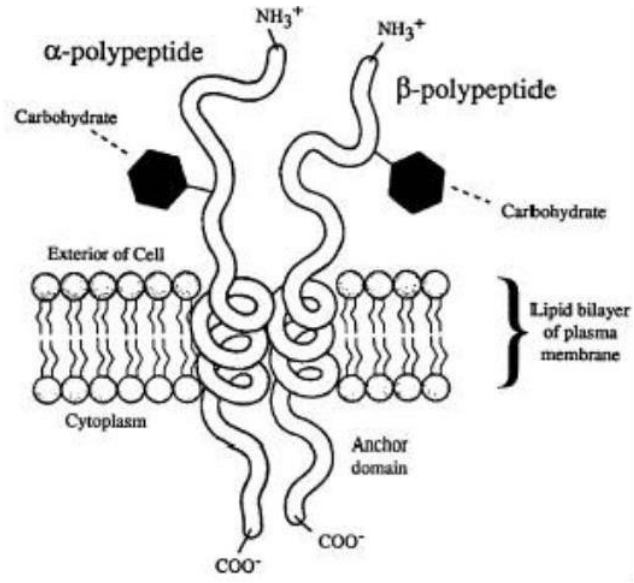

Figure 5 Structure and Orientation of an MHC Class II Glycoprotein Molecule (Robert A. Freitas Jr., 1999)

A.2.3 HLA Polymorphism

Each of the genetic loci can be expressed in different forms and is called an allele due to differences in nucleotide sequences within the coding regions. The HLA class I and class II loci are highly polymorphic with many hundreds of alleles. There are four hypotheses which have been advanced to try to explain this: a high mutation rate, gene conversion or interlocus genetic exchange, overdominant (balancing) selection and frequency-dependent selection. (Lawlor et al. 1990) Polymorphism is found predominantly in the $\alpha 1/2$ and $\beta 1$ domains of HLA class I and class II molecules, whereas The non-classical class I HLA-E, F and G and the class II genes not directly involved in antigen binding, HLA-DM and DO, are less variable. The numbers of alleles recognized at the classical loci assigned by the WHO Nomenclature Committee for Factors of the HLA System in the 14th International Histocompatibility Workshop in 2005, are presented in Table 1 (Marsh et al, 2005).

Table 1 The numbers of classical HLA alleles assigned by the WHO Nomenclature Committee in the 14th International Histocompatibility Workshop in 2005 (Marsh et al., 2005)

Locus	DNA-level Alleles	Serological Equivalents
HLA-A	349	40
HLA-B	627	88
HLA-C	182	9
HLA-DRB1	394	80
HLA-DQB1	61	7
HLA-DPB1	116	(-)

A.2.4 HLA Nomenclature

Different methods have been used over the years to analyze the HLA genes, leading to the complexity in nomenclature of the HLA system. Mixed Lymphocyte Culture is one of the earliest methods used, and HLA specificities were given names like Dw2. A later method was serologic typing (microlymphocytotoxicity), which using antibodies to identify the individual HLA molecules on the cell surface (eg. A, B, DR, DQ), which made it possible to assign Dw2 positive cells as bearing a DR2,DQ1 haplotype. Additionally, the DR2 specificities could be split into DR15 and DR16, similar to the DQ1 specificity that could be split into DQ5 and DQ6. Later characterization of HLA involved genomic methods (e.g. RFLP), the PCR-based methods and more recently DNA sequencing. The nomenclature is now based on the gene names followed by a star and an extension of the HLA allele names of four to eight digits to allow for up to 99 synonymous variants of each allele. The first two digits correspond to the serological specificity (a group of alleles), followed by the two digits representing the allele assignation (a specific HLA allele) and the next two digits express an allele which differs by a synonymous mutation.) The last two digits represent an allele which contains a mutation outside the coding region. Null or Low indicate the level of expression of the allele, but are rarely used. For instance, the variants of DR15 detected with serology have now been termed DRB1*150101 to DRB1*1516. (Marsh et al, 2005)

A. 3 HLA typing

Many techniques are used to determine the HLA type based on their phenotype or genotype. They can be classified into 2 categories, the serological methods (flow cytometer, lymphocytotoxicity test) and DNA-based typing methods (PCR-based methods, sequence based typing). For many years, HLA antigens were typed using sera containing antibodies of the Class I or II proteins, collected from multiparous women, or individuals who had received multiple blood transfusions. In addition, HLA Class II polymorphisms were examined by T-cell responses in the Mixed Lymphocyte Reaction (MLR). Nowadays, advances in molecular biology techniques have provided for introduction of PCR-based methods for HLA typing. Although the PCR technology

has improved the capacity for detecting genetic differences and is applied more and more often., lymphocytotoxicity test is still being used as the standard in routine HLA typing for transplantation. The allelic variability can often be accurately investigated via DNA-based typing methods, are performed to determine the genotype.

A. 3.1 Serological HLA typing methods

A. 3.1.1 Microlymphocytotoxicity

The polymorphism of HLA antigens can be examined by the patterns of a panel of antisera depended on a suitable cell suspension and quality reagents. Lymphocytes are used because they express both class I and class II molecules. For typing HLA class II antigens, B cells are needed. There are two forms of reagents, monoclonal antibodies and alloantibodies. The alloantibodies are produced by various sensitization procedures. The major source of alloantibodies is multiparous women, with secondary sources being blood donors and transplant recipients. (Middleton et al., 1993) Monoclonal antibodies can be used instead of alloantibodies but they are directed solely at a single epitope. It is impossible to obtain monoclonal antibodies which can be used to detect all HLA antigens due to theirs complexity. This technique is limited by the availability of either monospecific antibodies or typing cells.

A. 3.1.2 Mixed Lymphocyte Culture (MLC)

The mixed leukocyte culture detects class II antigens and measures the HLA-D compatibility between donor and recipient. The lymphocytes from 2 individuals are cultured together and the proliferative response (mixed lymphocyte reaction) is measured by 3H-TdR (radioactively labeled thymidine) uptake. Even though an MLC takes several days to complete, this test is an important in vitro test for studying allograft reactions. (Mickelson et al., 2000)

A. 3.1.3 Flow cytometry

Flow cytometry is the measurement of physical and/or chemical characteristics of single biological particles in a fluid stream passing through the path of one or more laser beams. The light scattered from these particles is detected and converted into an electronic signal corresponding to the relative size and complexity of a cell or particle. In addition to those two morphological parameters, several kinds of fluorescence can be measured per particle simultaneously. Flow cytometry is used to specify weak anti-HLA antibodies that are not normally detected by less sensitive methods and HLA antigens which are the specific targets of those antibodies. HLA typing by flow cytometry is performed as a lysed whole blood technique using a single color, directly conjugated antibody and gated peripheral blood lymphocytes as the marker population. (Gilman N.,1993)

A. 3.2 DNA-based HLA-typing methods

Although serology is a quick and convenient method, it is hindered in many cases by serological cross-reactivity and decreased expression of HLA antigens. Advances in molecular technology have led to changes in methodology for HLA typing. There are significant correlations between HLA class II phenotyping and PCR-based HLA typing (Bidwell et al., 1994).

A. 3.2.1 Restriction fragment length polymorphism (RFLP)

The restriction fragment length polymorphism (RFLP) technique relies on the ability of a specific restriction endonuclease to distinguish exact DNA nucleotide sequences by cutting the DNA at each of these points into restriction fragments that differ in size from each other. RFLP is performed by digesting DNA from different individuals with a restriction enzyme, followed by gel electrophoresis and in-situ denaturation of duplex DNA followed by transfer to a support membrane, then visualized by hybridization with homologous radiolabelled genomic or cDNA probes. (Bidwell, 1994). Many investigators prefer the restriction enzyme Taq I because it permits distinction between the majority of DRB-DQB-DQA haplotypes (Bidwell et al., 1994).

Variation in nucleotide sequences between HLA alleles is reflected by allele-specific hybridization signal patterns. The disadvantages of this method are that it takes approximately two weeks to achieve a complete HLA class II typing and the need for radioactive labeling of probes.

A.3.2.2 Polymerase chain reaction (PCR)

The polymerase chain reaction, developed in 1980, is a technique for the in vitro amplification of a desired sequence of DNA that depends on the extension of primers annealed to opposite strands of denatured double-stranded DNA template at double-stranded temperatures. There are three steps involved in the PCR reaction, denaturation, annealing and polymerization. Denaturation to promote single-strand DNA is achieved by heating DNA templates to approximately 95°C, then reducing the temperature significantly to allow base-pairing (annealing) of the primer to the target DNA. The temperature is then changed to the optimum temperature in order for the DNA polymerase enzyme to catalyze extension of the complementary primers to the template. (polymerization) By repeating the melting, annealing and extension steps, a large quantity of the original template DNA can be generated. (Rabinow, 1996)

There are several factors that can affect PCR specificity. Denaturation time and temperature are the most likely sources of failure to generate a PCR product. Two complementary sequences form hydrogen bonds between their complementary bases (G to C, and A to T or U) and form a stable double-stranded, anti-parallel molecule. By heating to a point above the melting temperature, single-strand molecules can be produced. The melting temperature is generally less than 100°C if the nucleic acid is heated in buffers of ionic strength lower than 150mM NaCl. Additionally, the half-life of the Taq DNA polymerase is given as having a half-life of 30 min at 95°C and less than 5 min at 98°C °, whereas other enzymes may have longer or shorter half-lives. Therefore denaturation time and temperature should be considered as well as the number of PCR cycles when selecting a thermostable enzyme to use. Another critical important parameters of a successful amplification are primer length and sequence. The melting temperature of a double-stranded nucleic acid increases both with its length and with increasing (G+C) content. A simple formula for calculation of the melting temperature (Tm) is $Tm = 4(G + C) + 2(A + T)$ °C.

Therefore, the annealing temperature (Ta) depends directly on length and sequence of the primer and can be approximated for oligonucleotides of 25 bases or less as follows: Ta = Tm - 5°C (Innis and Gelfand, 1990). The Ta is increased by 1°C every other cycle, and specificity of amplification and yield of products are both increased. (Rychlik et al. 1990) On the other side of the equation, similar or related targets can be amplified by lowering the Ta and then one or both primers will anneal to other sequences than the true target. Internal single-base mismatches or partial annealing may be tolerated. However, this can lead to non-specific amplification and consequent reduction in yield of the desired product, if the 3'-most base is paired with a target. Properly designed primers should contain 15-30 nucleotides in the complementary region (3'-end of the molecule), whereas 4-30 bp in non-complementary 5' tails or linkers are fine. Too-long primer length may promote mismatch pairing and non-specific priming due to an increase in annealing temperatures. The designed primers should have a random base distribution with average GC content. AT-rich, GC-rich regions and internal secondary structures should be avoided if possible. Ideally, GC-content should be 40-60%. The elongation temperature of PCR depends on the Taq DNA polymerase, whereas elongation time depends both on the DNA polymerase and the length of the DNA fragment to be amplified, optimally at 70 - 72°C, for 0.5 - 3 min (1 minute per thousand base pairs) (Innis and Gelfand, 1990). Another consideration for PCR optimization is the reaction buffer. Recommended buffers generally contain 10-50 mM Tris-HCl pH 8.3, up to 50 mM KCl, 1.5 mM or higher MgCl2(Mg is a required cofactor for thermostable DNA polymerases), 0.2 – 1 μM each primers , 50 – 200 μM each dNTP, gelatin or BSA to 100 ug/ml, and/or non-ionic detergents such as Tween-20 or Triton X-100 (0.05 - 0.10% v/v) (Innis and Gelfand, 1990). Primer concentrations should not be higher than 1μM unless there is a high degree of degeneration. Magnesium concentration is an essential factor that can affect the success of the PCR by reducing enzyme fidelity in case of excess free magnesium (Eckert and Kunkel, 1990) and may increase the rate of nonspecific amplification (Ellsworth et al. 1993). The magnesium concentration should be 0.5 - 2.5mM greater than the dNTP concentration. A buffering agent, most often a Tris-based buffer, and salt, normally KCl, modulates the pH of the reaction, which influences the enzyme activity and fidelity. Some salt is necessary to facilitate primer annealing but higher concentrations than 50 mM can inhibit Taq polymerase. The recommended Taq DNA polymerase concentration is 1–1.25 units of in a 50μl amplification

reaction. Adding more enzymes will not significantly increase product yield but can generate artifacts (smeared bands in an agarose gel) that are associated with the intrinsic $5' \longrightarrow 3'$ exonuclease activity of Taq DNA polymerase. (Longley et al. 1990; Bell and DeMarini, 1991). DNA template quantity and quality are also involved in the success of amplification. Contaminants in nucleic acids can inactivate DNA polymerases. Reactions with too little DNA template will have low yields, while reactions with too much DNA template can be plagued by nonspecific amplification. The number of amplification cycles required for promoting a band visible on a gel depends on the starting concentration of the template. Innis and Gelfand (1990) recommend from 40 - 45 cycles to amplify 50 target molecules, and 25 - 30 to amplify 3x105 molecules to the same concentration because of plateau effect (an attenuation of the normally exponential rate of product accumulation in a PCR reaction). It can be caused by depletion of dNTPs and primers, stability of the reactants (e.g. enzyme activity), end product inhibition by duplex DNA non-specific competition for resources and reannealing of specific products to one another instead of to the primers. In addition, some reagents can be added to enhance the PCR by increasing the yield of the desired PCR product or decreasing the production of undesired products, for instance betaine, DMSO and formamide can be helpful when amplifying GC-rich templates and templates that form strong secondary structures. (Rees *et al*. 1993). In some cases, a failure to amplify a region of DNA can be overcome by adding a general stabilizing agent such as BSA (0.1mg/ml), gelatin (0.1–1.0%) or a nonionic detergent (0–0.5%).

A.3.2.2.1 Polymerase chain reaction-sequence-specific oligonucleotide (PCR-SSO)

Polymerase chain reaction-sequence specific oligonucleotide (PCR-SSO) typing was the first PCR-based test to offer substantial improvements on RFLP. A whole region of target DNA (for example the HLA DR gene region) is amplified in the PCR. The target DNA sequences are commonly amplified from exons 2 and 3, with or without intron 2 of HLA class I genes and exon 2 of HLA class II genes which are polymorphic (Bidwell, 1994). The amplified DNA is then tested by adding a panel of SSO probes (designed and standardized during the 11th international

histocompatibility workshop) which are complementary to a different motif within the hypervariable region (HVR) of HLA sequences. Specific patterns of SSO probe hybridization are used to characterize certain HLA antigens. There are mainly 2 formats of PCR-SSO hybridization. The first format, based on the membrane solid phase, is the solid phase dot-blot, slot blot and reverse dot-blot. Recently, this method has been modified for use in large scale studies. The second format, based on the microtiter tray, is the oligocapture sandwich assay and the dual-phase oligocapture assay. (Bidwell, 1994)

A.3.2.2.2 Polymerase chain reaction-sequence-specific primer (PCR-SSP)

PCR-SSP is based on the principle that a completely matched primer will be more efficiently used in the PCR reaction than one with one or several mismatches. This test is used in amplifying a target sequence rather than a mismatched oligonucleotide primer by recombinant Taq polymerase. The primer is designed to have a perfect match with only a single allele or group of alleles. Under strictly controlled PCR conditions, perfectly matched primer pairs result in the presence of a specific amplified DNA fragment. (positive result) while mismatched primer pairs, particularly at the 3'end of the primer, result in the absence of a specific amplified DNA fragment (negative result). Typing is done by using a set of different PCR's, each with primers specific for different HLA antigens. (Bunce et al., 1995) Due to adverse effects that can be caused by various factors such as the presence of inhibitors, poor DNA quality, etc., an internal control primer pair is included in every PCR reaction. The control primer pair amplifies a conserved region of a human gene present in all human DNA samples such as the Growth Hormone or the Hemoglobin gene. The procedure consists of three steps: DNA isolation, PCR amplification and visualization of the PCR product under UV light. Identification of HLA loci by PCR-SSP has proved to be a rapid and accurate method for genotyping HLA-A, -B, -C –DR and -DQ alleles. (Otten et al., 1995) (Welsh and Bunce, 1999)

A. 4 HLA and diseases

It has been known for some time that certain diseases are associated with certain HLA alleles. Previous studies have shown that more than 100 diseases are found more frequently in individuals bearing certain HLA alleles or haplotypes than in the general population. (Svejgaard et al., 1996). Most diseases that show association with particular HLA genes have a prominent autoimmune character such as some of the infectious diseases, inflammatory diseases and autoimmune diseases. The immune response genes of the HLA region are influence resistance or susceptibility to disease however, the mechanism remains imprecisely defined. The involvement of HLA molecules in the selection of the T-cell repertoire in the thymus and activation of an immune response by presentation of peptides outside the thymus likely underlie this phenomenon. Protective HLA gene alleles may mediate the elimination of potentially pathogenic T-cells in the thymus, whereas susceptible HLA gene alleles may fail to contribute. In addition, HLA genes are involved in selective binding of autoantigenic peptides and expansion of autoreactive T-cells (Carson, 1992). Another possibility is that disease-causing genes are closely linked to a particular HLA allele and they are inherited together, e.g. the haemochromatosis and 21-hydroxylase deficiency-causing genes. (Feder et al., 1996) DM, TAP, TNF, LMP and complement are another possible candidate genes that may influence the immune response. (Djilali-Saiah et al., 1996) HLA genotypes can also underlie responsiveness or unresponsiveness to certain vaccines. For instance, the incidences of unresponsiveness to vaccination with hepatitis B surface antigen of subjects who are HLA-DR3 have considerably increased. (Peces et al., 1997)

Different diseases are associated with different HLA alleles in different ethnicities, e.g. rheumatoid arthritis (RA), insulin dependent diabetes mellitus (IDDM), thyroiditis, Goodpasture's syndrome, ankylosing spondylitis, myasthenia gravis and multiple sclerosis (Stastny et al., 1983) (Table 2) For example, HLA-DR3 and HLA-DR4 are crucial susceptibility markers in RA and IDDM among Caucasians (Segall, 1988; Winchester et al., 1994),

However, there are patients with other HLA haplotypes that also have the disease and the diseaseassociated haplotypes are also found in some healthy individuals. So these diseases might have several genetic components of which HLA is but one factor.

Table 2 HLA-associated diseases and phenotypes (Shiina et al., 2004)

Phenotype or disease	Disease class	Gene symbol
Acute anterior uveitis	IMMUNE/OCULAR	HLA-B27, MICA
Acute graft-vs-host disease (GVHD)	IMMUNE/TRANSPLANTATION	HLA
Alopecia areata (AA)	IMMUNE/DERMATOLOGY	NOTCH4
Alpha-1-anti-trypsin deficiency	RESPIRATORY	HLA
Alveolar echinococcosis (AE)	INFECTION/PARASITOSIS	HLA-DRB1
Alzheimer's disease	NEURODEGENERATIVE	HLA-A2, -DRB1
Ankylosing spondylitis (AS)	IMMUNE/RHEUMATOLOGY	HLA-B27, TNF
Aortoarteritis	VASCULAR	HLA-DRB1
Asthma	IMMUNE/RESPIRATORY	HLA-DRB1, -DQB1, -DPB1
Asthma. total IgE	IMMUNE/RESPIRATORY	HLA-DR2, -DR4, -DR7
Atopy	IMMUNE	HLA-DR4, -DR7, -DQB1, -DRB1
Autism	PSYCH	HLA-DRB1
Autoimmune hepatitis	IMMUNE	HLA-DRB1
Autoimmune pancreatitis	IMMUNE	HLA class-II
Autologous mixed lymphocyte reaction	IMMUNE	HLA-DR
Behcet's disease	IMMUNE/OCULAR	HLA-B51, MICA
Bone marrow transplant	IMMUNE/METABOLIC	HLA-DPB1
Cardiac sarcoidosis	CARDIOVASCULAR	HLA-DQB1
Celiac disease	IMMUNE/GASTROINTESTINAL	HLA class-II, HSPA1A
Cervical cancer	CANCER/GYNAECOLOGY	HLA class-II

Phenotype or disease	Disease class	Gene symbol
Cervical intraepithelial neoplasia (CIN)	INFECTION/GYNAECOLOGY	HLA DQB1
Childhood acute lymphoblastic leukaemia (ALL)	CANCER	HLA-DR
Childhood diabetes	METABOLIC/ENDOCRINE	HLA
Chronic hepatitis C	INFECTION/LIVER	HLA class-II
Complement C4 complotypes	IMMUNE	HLA-B17
Congenital adrenal hyperplasia (CAH)	METABOLIC	CYP21
Crohn's disease	IMMUNE/GASTROINTESTINAL	HLA-DRB1, TNF
Cyclosporine-dependent aplastic anaemia (CyA-dependent AA)	IMMUNE/HAEMATOLOGY	HLA class-II
Defective DR beta 4 chain expression to the HLA-DRB1 gene	IMMUNE	HLA-DRB1
Delayed sleep phase syndrome (DSPS)	PSYCH/NEUROLOGICAL	HLA-DR1
Dermatomyositis/ polymyositis (DM/PM)	IMMUNE/MUSCLE/DERMATOLOGY	HLA-DQB1
Dystrophic epidermolysis bullosa (RDEB)	DERMATOLOGY	HLA
Endometriosis	GYNAECOLOGY/FERTILITY	HLA-DQA1, -DPB1
Familial juvenile onset psoriasis	IMMUNE	HLA class-I
Gastric adenocarcinoma	CANCER	HLA-DQB1
Giant cell arteritis (GCA)	VASCULAR/RHEUMATOLOGY	HLA-DR4
Graves' disease (GD)	IMMUNE/ENDOCRINE	HLA-DRB1, -DQA1
Hashimoto's thyroiditis	IMMUNE/ENDOCRINE	HLA-A2
High-serum IgE concentrations	IMMUNE	HLA-DRB1*01
HIV-related disease	IMMUNE/INFECTION	HLA-DR, -DQ

Phenotype or disease	Disease class	Gene symbol
HLA-DQA1 differential expression	IMMUNE	HLADQA1
Host defense and inflammatory	IMMUNE	HLA
Human T-cell lymphotropic virus type-I-associated myelopathy (HAM)	IMMUNE	HLA-DRB1, TNF
Hypertrophic cardiomyopathy	CARDIOVASCULAR	HLA
Idiopathic progressive sensorineural hearing loss (PSHL)	AUDITORY	HLA class-II
IgA deficiency	IMMUNE	HLA-A28, -B14
IgA deficiency and common variable immunodeficiency	IMMUNE	HLA-DQB1
IgA nephropathy (IgAN)	IMMUNE/UROLOGY	C4
Immotile cilia syndrome (ICS)	RESPIRATORY	HLA
Immune response to hepatitis B (HBV)	IMMUNE/INFECTION	HLA-DRB1
Increased cutaneous melanoma risk	SKIN CANCER	HLA-DQB1
Increased prevalence and level of insulin autoantibodies	IMMUNE/ENDOCRINE	HLA-DR4
Inflammatory bowel disease (IBD)	IMMUNE/GASTROINTESTINAL	HLA-DPA1
Juvenile ankylosing spondylitis (JAS)	IMMUNE/RHEUMATOLOGY	HLA class-II
Juvenile dermatomyositis (JDMS)	IMMUNE/VASCULAR/MUSCLE/SKIN	HLA-DQA1
Juvenile multiple sclerosis (JMS)	NEURODEGENERATIVE	HLA-DR2
Juvenile myoclonic epilepsy (JME)	NEURODEGENERATIVE	HLA-DRw6
Latex allergy	IMMUNE	HLA-DR4
Leprosy phenotypes	IMMUNE/INFECTION	HLA class-II, TNF
Low responsiveness to hepatitis B vaccine	IMMUNE/INFECTION	HLA-DR14-DR52

Phenotype or disease	Disease class	Gene symbol
Marrow graft rejection	IMMUNE/TRANSPLANTATION	HLA
Mediterranean visceral leishmaniasis (MVL)	INFECTION/PARASITOLOGY/IMMUNE	HLA-class-II, class-III
Minimal change nephrotic syndrome	RENAL/UROLOGIC	HLA-DRw8 and DQw3
Mixed cryoglobulinaemia (MC)	IMMUNE	HLA-DR
Mite-sensitive asthma	IMMUNE	HLA class-II
Multiple myeloma	CANCER	HLA
Multiple sclerosis (MS)	IMMUNE	TNF
Myasthenia gravis (MG)	IMMUNE	HLA class-II
Nasopharyngeal carcinoma	CANCER	HLA
Narcolepsy	NEUROLOGICAL	HLA class-II
Non-familial idiopathic dilated cardiomyopathy (IDC)	CARDIOVASCULAR	HLA-DRB1
Non-responsiveness to mellitin: a component of bee venom	IMMUNE	HLA class-II
Ocular cicatricial pemphigoid (OCP)	IMMUNE/ORAL/OCULAR	HLA-DR4
Pathological myopia (PM)	OCULAR	HLA-DPB1
Pemphigus vulgaris	IMMUNE/DERMATOLOGY	HLA DQ beta
Periodontitis	IMMUNE	HLA
Pigeon breeder's lung	IMMUNE	HLA-DR3
Pre-eclampsia	HYPERTENSION/CARDIOVASCULAR	HLA-G
Primary IgA nephropathy	IMMUNE/RENAL	HLA-DQ
Primary biliary cirrhosis (PBC)	CHOLESTATIC LIVER DISEASE	HLA-DPB1
Primary sclerosing cholangitis	BILE DUCT/LIVER	HLA-B8

Phenotype or disease	Disease class	Gene symbol
Prostate cancer	CANCER	HLA-A
Psoriatic arthritis (PsA)	IMMUNE/DERMATOLOGY/RHEUMATOID	HLA-B/C, MICA
Pulmonary tuberculosis (PTB)	INFECTION/RESPIRATORY	HLA-DRB1
Recurrent oral ulcers (ROU)	ORAL	HLA-DR3 or DR7
Rheumatoid arthritis (RA)	IMMUNE/RHEUMATOLOGY	HLA-DRB1, TNF
Schistosomal worm antigen	INFECTION	HLA-Dw12
Schizophrenia	PSYCH	HLA
Semple rabies vaccine-induced autoimmune encephalomyelitis (SAE)	IMMUNE/VACCINATION	HLA class-II
Serum testosterone level	METABOLIC/ENDOCRINE	HLA
Specific IgE	IMMUNE	HLA-DRB3
Skin prick test (house dust mite)	IMMUNE	HLA-DRB1
Skin prick test (cockroach)	IMMUNE	HLA-DRB1, -DQB1
Sudden infant death (SID)	CARDIOVASCULAR	C4?
Susceptibility or resistance to immunodeficiency virus type 1 infection	IMMUNE/INFECTION	HLA class-I
Systemic lupus erythematosus (SLE)	IMMUNE/RHEUMATOID	HLA class-II
Systemic scleroderma	IMMUNE/DERMATOLOGY/RHEUMATOID	HLA
Systemic sclerosis (SSc)	IMMUNE	HLA class-II
Takayasu's arteritis	CARDIOVASCULAR	HLA-DRB1
Thyroid carcinoma	CANCER/ENDOCRINE	HLA-DR1
Total IgE	IMMUNE	HLA-DRB1, -DQB1

Phenotype or disease	Disease class	Gene symbol
Tuberculosis (TB)	INFECTION/RESPIRATORY	HLA-DQB1
Type-1 diabetes mellitus (IDDM)	IMMUNE/ENDOCRINE/METABOLIC	HLA-DRB1, TNF
Type-2 diabetes mellitus	IMMUNE/ENDOCRINE/METABOLIC	HLA class-II
Ulcerative colitis (UC)	IMMUNE	MICA
Viral clearance	INFECTION/IMMUNE	HLA-DR13
Vogt-Koyanagi-Harada syndrome (VKH)	IMMUNE/OCULAR	HLA-DR4
Young-onset keratoconus	OCULAR	HLA
X-linked adrenoleukodystrophy (X-ALD)	METABOLIC	HLA-DRB1

B. Graves' disease (GD)

Graves' disease (GD), also known as "diffuse toxic goiter", is a thyroid-specific autoimmune disorder caused by thyroid-stimulating immunoglobulin(TSI), which the body produces against the thyroid-stimulating hormone receptor (TSH-R) on thyroid cells, leading to an abnormally strong release of hormones from the thyroid gland (hyperthyroidism), characterized by clinical hyperthyroidism with diffuse goiter, ophthalmopathy in many cases and detecting of autoantibodies against the thyroid-stimulating hormone receptor (TSH-R) on thyroid cells. Graves' disease, named after Robert James Graves for his description in 1835 (Gossage et al, 1991), is one of the most frequent and serious organ-specific autoimmune diseases, besides IDDM and Myasthenia Gravis, and involves a complex interaction of multiple genetic, environmental and endogenous factors. (Weetman, 1994). Graves' patients most commonly present with a diffusely enlarged thyroid gland from lymphocytic infiltration and hyperthyroidism. Ophthalmopathy, either infiltrative or non-infiltrative, and dermopathy are less common expressions. Finger clubbing and acropachy can also be found (Feliciano, 1992, McDougall, et al., 1991). GD is more prevalent in white and Asian populations than black populations and in females more than males with the highest-risk ages for either sex being between 40 and 60 years (Weetman, 2000). Thus, as yet

unidentified age-related factors and/or hormonal changes may contribute to enhanced susceptibility.

Regarding hyperthyroidism, 25–50% of GD patients develop a clinical feature of the eyes known as thyroid-associated ophthalmopathy (TAO) or Graves' ophthalmopathy (GO) Noninfiltrative GO is caused by stimulation at the levator muscle of upper eyelid which causes the upper eyelid to retract. Infiltrative GO is due to mucopolysaccharide deposition and infiltration of the orbit and extraocular muscle. (Burch and Wartofsky, 1993) Skin changes occur in 5% to 10% of patients and usually coexist with ophthalmopathy. The deposition of mucopolysaccharides is the cause of dermopathy, especially in the pretibial area (Feliciano, 1992). Acropachy is present in less than 1% of patients. In rare cases, patients with GD develop optic neuropathy with visual defects, and, rarely, blindness.

Although the diagnosis of Graves' disease may be difficult in patients with atypical manifestations, laboratory tests of thyroid autoimmune disease markers such as triiodothyronine (T3), thyroxine (T4) and suppressed thyrotropin (TSH) levels can be used to confirm the physical examination (Feliciano, 1992; McDougall et al., 1991).

B. 1 Etiopathogenesis of Graves' disease

Predisposition to Graves' disease is supposed to be multifactorial, even though its etiology is not completely clarified. (Weetman et al., 1994). Infectious, environmental, immune and genetic factors are likely to be involved in the development of the disease. (Brix et al., 1998) The ingestion of iodine, infectious agents and stress are nongenetic factors that are often involved in pathogenesis of disease. Incorporation of iodine, a necessary component of normal thyroid hormonogenesis, into thyrosine residues of thyroglobulin, the main protein synthesized in the thyroid, results in the formation of mono-iodotyrosine and di-idothyrosine derivates that lead to the production of T3 and T4. Several studies have suggested that iodination of thyroglobulin is crucial for recognition by thyroglobulin-reactive T cells. (Rasooly et al., 1998) (Champion et al., 1991) There are studies that suggest a highly iodinated thyroglobulin molecule is a better immunogen than a lesser iodinated one and such molecules may assist in antigen uptake and

processing by APC. (Ebner et al., 1992) The ingestion of iodine is correspondingly associated with the lymphocytic infiltration of the thyroid gland and the detectable thyroid autoantibodies in circulation. In one study, cross-reactive antigens caused the production of thyroid autoantibodies capable of reacting with TSHR in Graves' patients (Weetman et al., 1994). Molecular Mimicry, a theory on the etiology of autoimmune diseases, the theory that exposure to a particular peptide epitope in an environmental antigen might raise immune reactivity to an amino acid sequence identical to that present in an human endogenous antigen, is one of the most commonly studied mechanisms for the induction of autoimmunity. The entire immune system, including T and B lymphocytes, monocytes, granulocytes and humoral factors, participate in the autoimmune process (Gergely, 1992). The alteration of the immunological mechanism of all autoimmune diseases represents a failure to maintain immune tolerance to self antigens (Herold et al., 1995). It is well known that B and T lymphocytes, the effectors of the immune system, can be divided into T-helper (Th) cells, which express CD4+ surface antigens, and T-cytotoxic (Tc) cells, which express CD8+surface antigens. The CD4+ Th precursor cells are further subdivided into two populations, Th1 and Th2 cells. Th1 cells secrete interleukin-2 (IL-2), interferon g(IFNg) and tumour necrosis factor alpha (TNFa),which modulate the cellular-mediated immune response and the initiation of tissue damage. Th2 cells secrete interleukins, IL-4, IL-5, IL-6 and IL-10, and are activated to provide help to B lymphocytes for specific immunoglobulin (Ig) production. Lymphocytes develop from the precursors in the bone marrow and mature in the thymus, where they are educated to recognize endogenous epitopes, and thus to generate self-tolerance against these antigens. (Peter Parham, 2005) Lymphocytes which strongly react with endogenous epitopes presented by HLA molecules and fail to recognize endogenous HLA molecules undergo negative selection resulting in an apoptosis of 95 - 98% of lymphocytes developing in the thymus.(Strominger, 1989)(Abbaset al., 1994) Presumably, in a developmental process designed to provide the maximum repertoire of lymphocytes, some lymphocytes which weakly recognize autologous antigens in the context of autologous HLA are allowed to persist in the circulation. Whether this varies from person to person and is involved in the selection for Graves' disease is unknown.

In 1956, Adams and Purves found a factor that caused stimulation of animal thyroid for a longer time than thyroid-stimulating hormone (TSH) reactivity, in the serum of hyperthyroidism patients. (Adams et al., 1956). This factor,Long-Acting Thyroid Stimulator, LATS, belonging to the IgG class, was proven to be a thyroid-stimulating immunoglobulin (TSIg or TSAb). It mimics the action of TSH by binding to amino acid positions 25-30 at the amino terminal of the extracellular region of the thyroid stimulating hormone receptor (TSHR) (Nagayama et al., 1992), causing excess secretion of thyroxine and triiodothyronine. TSIg can be detected in the majority of patients (77.8%) with Graves' disease. (Macchia et al., 1989) A two-stage process is involved in thyroid autoimmunity; the first stage sees the increased appearance of intrathyroid antigen presenting cells (APC) that carry and present thyroid autoantigens to Th cells, and in the second, the lymphocytes interact with the presented autoantigens, leading to the propagation of a large number of autoreactive CD4+Th lymphocytes,CD8+Tc lymphocytes and antibody-producing B lymphocytes that infiltrate the thyroid parenchyma. The interaction between the thyrocytes and infiltrating lymphocytes result in the different clinical outcomes of AITD.

Environmental factors may involved in pathogenesis by causing a reduction in generalized suppressor T cells and then reducing suppression of the thyroid-directed helper T cell (Th) population. In the presence of monocytes and the specific antigen, the specific Th cells produce interferon-γ (IFN-γ) and also stimulate specific B cells to produce TSIg (Volpé, 1991). Th1 cells secrete IL-2, IFN-γ and tumor necrosis factor (TNF), contributing to the pathogenesis of IDDM and experimental autoimmune encephalomyelitis (EAE) (Liblau et al., 1995). IL-2 production by PHA stimulation was reported to be reduced in Graves' PBMC cultures (Eisenstein et al., 1994). Conversely, another report showed significant enhancement of IL-2 production, proliferative response to mitogenic stimulators and the expression of HLA-DR antigen in untreated T-cells derived from Graves' disease patients compared to those of treated patients and healthy controls (Marazuela et al., 1994). Ropars also found that two anti-human thyrotropin receptor monoclonal autoantibodies, 11E7 and 34A, induced, with an intensity comparable to that of IFN-gamma, the up-regulation of MHC mRNA and proteins in humans and murine thyroid epithelial cells (Ropars et al., 1994). This can lead to an increased efficiency of the presentation of autoantigens and activation of autoreactive T cells.

The influence of genetic factors in the etiology of disease is well established. Studies of pairs of twins suggest that genetic factor involvement in the development of Graves' disease is as high as 79%, with environmental factors accounting for the remainder. Studies involving twins indicate a higher degree of concordance. Identical siblings had a 50% chance of developing GD when their other one had the disease. The risk was 30% for nonidentical twin (McDougall et al., 1991). Although many of the genetic contributions remain elusive, human leukocyte antigen (HLA), tumor necrosis factor (TNF-β), cytotoxic T-lymphocyte-associated protein 4 (CTLA4), and thyroid stimulating hormone receptor (TSHR) have been shown in association studies to confer susceptibility to GD (Weetman et al., 1994)., but only two loci , the humanleukocyte antigen (HLA) region on chromosome 6p21 and the cytotoxic T lymphocyte–associated 4 gene (CTLA4) on chromosome 2q33 have been confirmed as playing crucial roles in the susceptibility to GD. (Ueda et al. 2003). HLA class I and II genes appear to contribute to autoimmunity in general. The HLA genes encode for proteins essential in the defense mechanism against disease-causing pathogens by discriminating 'self' from 'non-self' antigens, therefore the error occurs in this pathways with contributions of gene defects, and environmental factors may cause an immune system mistake by attacking the body instead of the pathogens, resulting in an autoimmune disease.

HLA class III and other non-HLA genes such as TNF, HSP70, TAP (transporters associated with antigen processing), LMP (large multifunctional protease), DMA and DMB are also critical to the immune response. It is possible that HLA associations as seen in thyroid autoimmunity are due partially to genetic variation in these closely linked immune regulatory genes and their linkage disequilibrium with class I and II genes (Makhatadze NJ, 1998)

B. 2 HLA antigens associated with Graves' disease

The first genetic factor to be associated with Graves' disease was HLA-B8. (Grumet et al., 1974) Subsequently, this relation was found to be more specifically with an MHC Class II molecule, HLA-DR3, due to strong linkage disequilibrium (LD) between HLA-B8 and HLA-DR3. (Farid et al., 1980) However, the majority of studies since then have focused on the HLA class II region. In contrast, inheritance of HLA DRB 1*07 appears to be protective. (Chen et

al., 1999) The thyroid gland section and the thyroid cell cultures from Graves' thyroidectomy specimens showed deviant HLA-DR expression (Hanafusa et al., 1983). This may be due to stimulation by local immune response because it is known that MHC class II mRNA and antigens of thyroid cells can be upregulated by IFN-γ (Bottazzo et al., 1983). The expression of HLA-DR antigens was detectable in most cases, followed by HLA-DP, and HLA-DQ on thyrocytes (Bottazzo et al., 1986).

In 1974 Grumet and colleagues found an association between HLA antigen B8 with Graves' disease in the American population. (Grumet et al., 1974). Later, the HLA-DR3 antigen was found to be related with Graves' disease in other Caucasian populations (Dahlderg et al., 1981; Farid et al., 1979; Schlensener et al., 1983) and it was also claimed to be a specific marker for those who relapse after a course of antithyroid drugs (McGregor et al., 1980). This DR3 antigen is thought to be the most specific marker of Graves' disease susceptibility. The HLA-DQA*0501 was found to be associated with GD in Caucasians. (Yanagawa et al. 1994) and (Badenhoop et al. 1995). Conformation of a stronger independent association of DQA1*0501 with GD was shown by the disease still being present in DR3 negative subjects. Different from Caucasian populations, the HLA-DR3 allele was not found to be associate with GD in several Oriental populations studies. The previous study, conducted on Thai GD patients in King Chulalongkorn Memorial Hospital , Bangkok, suggested that DRB1*1602-DQA1*0102-DQB1* 0502 haplotype was significantly increased in GD patients (P=0.0209,OR=2.55),DRB1*07-DQA1 *0201 - DQB1*0201 haplotype (P=0.039, OR=0.32) and HLA-DRB1*12-DQA1*0601 - DQB1* 0301 haplotype (P=0.0025, OR=0.28) were significantly decreased in GD patients. (Wongsurawat et al, 2006). A significant association of DRB1*1602-DQA1*0102-DQB1*0502 and HLA-DRB1*12-DQA1*0601- DQB1*0301 alleles and haplotypes with GD was recently reported in Korean studies, but has not been found in any Caucasian studies. (Wongsurawat et al, 2006). The researchers also suggested that the DRB1*1602-DQA1*0102-DQB1*0502, might serve as a marker for genetic susceptibility to GD in Asian populations. However, it remains unclear whether the primary susceptibility is the result of a haplotype or single locus effect, probably due to small sample groups and comparisons between differing geographical and ethnic backgrounds. Large sample sizes with accurate HLA subtyping may be one way in which this could be resolved.

Objectives

The purpose of the present study was to determine the frequency of different alleles at the HLA-DRB1 and HLA-DQB1 loci of Graves' patients at Songklanagarind Hospital in southern Thailand, a university hospital and the major tertiary care center in southern Thailand. It is hoped that this analysis will prove to be useful in understanding the role of the HLA gene in contributing to Graves' disease susceptibility, and in helping to pinpoint a genetic marker for susceptibility or resistance to Graves' disease.