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Human Leukocyte Antigens With Special Reference To Association And Linkage In Multiple Sclerosis

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SUMMARY

The polymorphism of HLA (human leukocyte antigen) is extensive. Allelic variation has previously been detected by cellular and serological methods. Today a variety of genomic typing techniques have been developed, which give higher resolution and a possibility to detect single nucleotide variations. One such HLA typing technique, PCR-amplification with sequence-specific primers (PCR-SSP), has been developed in our laboratory. The application of PCR-SSP for typing of the DQA1 locus and its use in the study of linkage and association in disease, is described in this thesis.

Better typing techniques have lead to identification of new alleles, of which one (DRB1*1415) is described here. The complexity of the HLA system is even more increased by the combination of alleles in different haplotypes. Tight linkage disequilibrium between the DR and DQ subregions ensures that specific DR and DQ alleles often are inherited together. The haplotype carrying the DQA1*0104 allele is described in this thesis.

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system. Its etiology is unknown, but probably MS is a multifactorial and polygenic disease with autoimmune characteristics. The only repeatedly verified genetic factor in MS is the HLA association with the class II haplotype DR2,DQ6,Dw2 (specified here with genomic typing as DRB1*1501,DRB5*0101,DQA1*0102,DQB1*0602). This haplotype is present in 60% of MS patients and 30% of controls in North European populations. The importance of HLA genes in MS has been questioned since it has been difficult to establish linkage with markers in this region. In Swedish multiplex families we do find significant linkage (lod score >3) between HLA-DR,DQ and MS.

Further analysis, with detailed PCR-SSP typing of all class I and class II loci (A, B, C, DR, DQ and DP) in 200 patients and 210 healthy controls, added to the complexity of HLA as part of the genetic susceptibility in MS. A*0301 was found to be positively associated with MS independently of DR2,DQ6,Dw2. In contrast, the A*0201 allele was underrepresented in the patient group and in DR2,DQ6,Dw2-positive individuals it decreased the relative risk from 3.6 to 1.8. In addition, the DPB1*0401 allele occurred in increased frequency together with DR2,DQ6,Dw2 in patients compared with controls.

In conclusion, HLA is probably one of the major genetic factors determining MS susceptibility, although several additional events, both genetic and environmental, are required to develop MS.

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(submitted)

CONTENT

ABBREVIATIONS

CTLA-4	**cytotoxic T lymphocyte antigen 4**
EAE	**experimental autoimmune encephalomyelitis**
H-2	**mouse MHC**
HLA	**human leukocyte antigen**
IDDM	**insulin dependent diabetes mellitus**
LMP	**low molecular weight proteasome**
LOD	**logarithm of the odds ratio**
MBP	**myelin basic protein**
MG	**myasthenia gravies**
MHC	**major histocompatibility complex**
MOG	**myelin oligodendrocyte glycoprotein**
MS	**multiple sclerosis**
RA	**rheumatoid arthritis**
RT-1	**rat MHC**
RFLP	**restriction fragment length polymorphism**
SLE	**systemic lupus erythematosus**
TAP	**transport-associated protein**
TcR	**T-cell receptor**

INTRODUCTION

The major histocompatibility complex (MHC) is the name that was given to a set of genes shown to play a key role in the rejection of transplants[1]. MHC is a general name for this complex in all species, but every species also has its special name for these set of genes and molecules. In mice it is referred to as H-2, in rats RT1 and in humans the human leukocyte antigens (HLA). This thesis is focused on the HLA complex and its association and linkage with disease, in particular multiple sclerosis.

The HLA molecules

Human leukocyte antigens (HLA) are membrane-bound molecules with a peptide binding part that interacts with the T-cell receptor (TCR)[2]. This event leads to the activation of the specific cellular immune response that protects us from invading microbes. HLA molecules are divided into class I and class II, which are distinct in structure, function and expression pattern.

HLA class I

The HLA class I molecules consist of an α-chain with three extracellular domains bound to β2-microglobulin chain (Fig 1). The crystal structure of the HLA class I molecule shows that the first and second domain of the α-chain forms a β-pleated sheet and two α-helixes, creating a groove in which peptides of around nine amino acids can bind[3-7].

HLA class I molecules interact with TcRs of CD8-positive, mainly cytotoxic, T cells[3-8]. HLA class I molecules are expressed on most nucleated cells in the body. Peptides presented by class I are mainly of endogenous origin and they are generated in the cytosol by a proteasome consisting partly of low molecular proteasome-proteins (LMP-proteins)[9-12]. These peptides are subsequently transported into the ER via the transporter-associated proteins (TAP), where they are loaded onto the class I molecules[13,14]. The presentation of endogenous peptides ensures that the immune system will find infected cells, which then can be eliminated by cytotoxic T cells.

There are three types of classical HLA class I molecules, HLA-A, -B and -C. The function of these three molecules is probably similar, and having more then one class I molecules ensurs more extensive presentation of peptides and thereby higher probability of interacting with suitable T cells. The HLA class I molecules also interact with different NK cell inhibitor receptors and thus protects healthy cells from an immune attack by the NK cells[15-17]. There is also the non-classical HLA-E and HLA-F molecules, which have limited expression[18], and the HLA-G molecules that are expressed only on the placenta, all three with as yet unclear function[19,20].

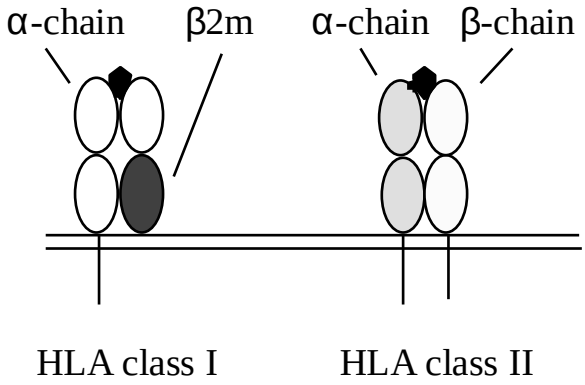

Figure 1. The HLA class I and class II molecules. HLA class I consits of a three-domain α-chain and the β2m chain. The class II molecule consist of one α-chain and one β-chain with two extracellular domains each. The black dots indicates where the peptides bind.

HLA class II

HLA class II molecules consist of one α- and one β-chain, which have two extracellular domains each (Fig.1). The first domain of the α- and the β-chain together form a structure of β-pleated sheet and two α-helixes, similar to that of the class I molecules, where slightly longer peptides of 10-25 amino acids[5, 21-24]. The crystal structures of the class II molecule shows that this structure is more open at the ends and hence the peptide can extend out from the binding groove[25-27].

Class II molecules interact with CD4-positive T cells, which mainly have a helper function. They are expressed on cells of the immune system, like macrophages, dendritic cells, B-cells and activated T-cells. Class II bound peptides are derived from a class II peptide-loading compartment that lyse with endocytosed vesicles, and thus these peptides are mainly of extracellular origin[10, 28-30]. The class II molecules are protected by the invariable chain (Ii) when they pass the ER[31, 32]. This chain is partly cut off by enzymes in the peptide loading compartment, leaving a CLIP fragment that the DM molecule replaces with peptide[33-39].

Three types of class II molecules are expressed and functional in the immune system, the DR-, DQ- and DP-molecules. An additional heterodimer of the DN and DO molecules is also expressed, but has unknown function. On B-cells, the most abounded molecules are the DR, followed by DQ (1/10 of the DR) and then DP (1/100 of the DR)[40].

The HLA genes

The HLA encoding region is a 3-5 Mb long stretch on the short arm of chromosome 6 (6p21.3), that is divided into the class I, class II and class III region. Many of other genes, in addition to the HLA genes, are encoded in this region, of which many have immunological functions (Fig. 2).

HLA class I genes

The class I region is a 1800 kb long part of the chromosome in which the α-chain of the HLA class I molecules are encoded. The β2-microglobulin is encoded on chromosome 15. The HLA-B locus is at the most centromeric side, followed by the HLA-C locus at less then 100 kb distance and the HLA-A gene at a distance of over 1400 kb from the B-locus. In between these loci are the genes of e.g. transcription factors and GTP binding proteins. The set of non-classical class I (HLA-E, -F and -G) and pseudogenes (HLA-H, HLA-J, HLA-K and HLA-L) is also present in the class I region[41].

The HLA class I genes consist of eight exons corresponding to the signal sequence, the three domains, the transmembrane region, the cytoplasmatic tail of the protein and the 3' untranslated region. The introns in-between vary in length from 33 to 599 base pairs.

The polymorphism of the HLA class I genes is extremely extensive, with 90 HLA-A, 188 HLA-B and 48 HLA-C alleles reported in June 1997[42]. Most of the polymorphism lies in the second and third exons, corresponding to the peptide binding part and TcR interacting part of the molecule, but there is also some polymorphism found in other exons[43].

HLA class II

The 800 kb long HLA class II region is located centromeric of the HLA class I region. Most telomeric is the gene for the α-chain of the DR-molecule, the *DRA* locus, followed by the genes for the β-chain encoded by the *DRB* loci[41]. There are several types of *DRB* genes.

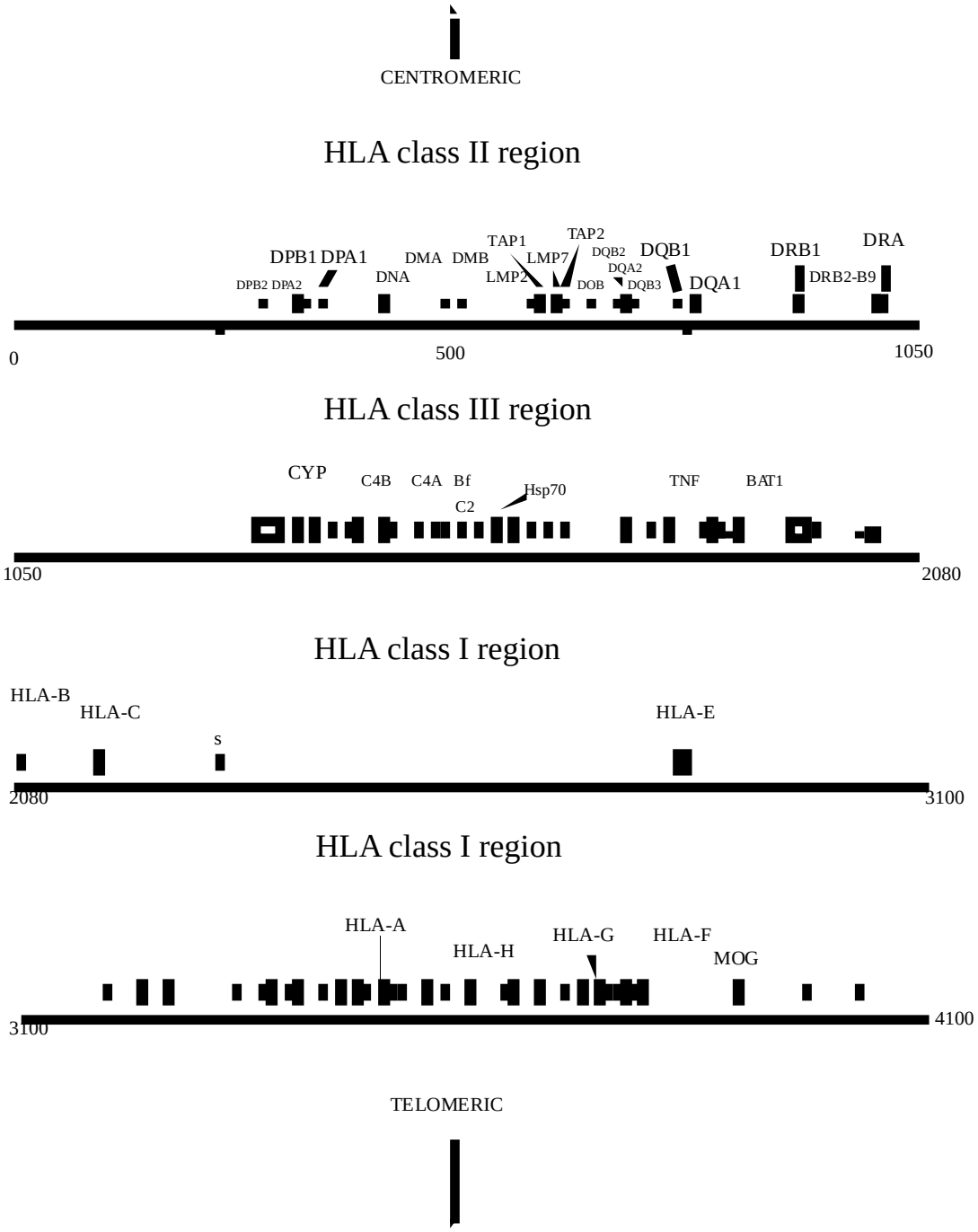

Fig. 2. The HLA class II, III and I region of chromosome 6. The genes for the DR, DQ, DP, DN and DO molecules are in the class II region as well as genes for peptide processing molecules (TAP, LMP, DM). The class III regions includes genes for complement, TNF and heat shock protein 70. The HLA-A, -B, -C, -E, -H and -G genes are found in the class I region.

All individuals carry at least one DRB1 gene and in addition most individuals also carry one or several DRB3, DRB4 or DRB5 genes depending on the haplotype (Fig. 3).

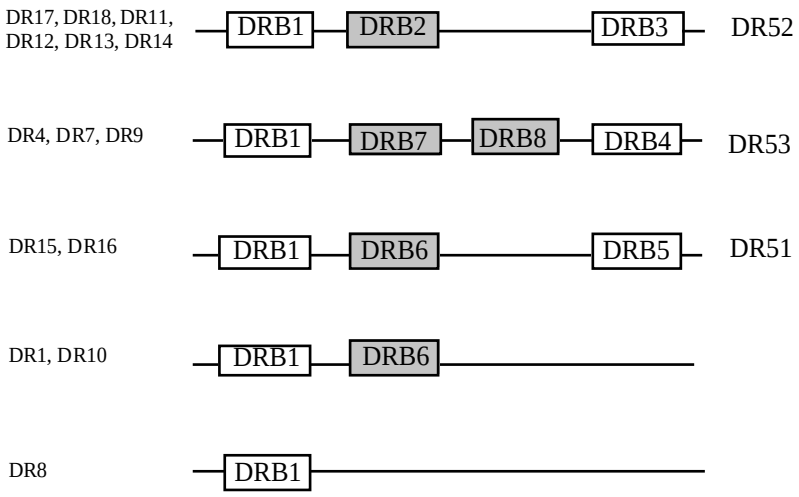

Fig. 3. DR haplotypes, shown on the left side, with the broad specificities shown on the right. Shadowed squares mark pseudogenes.

Fifty to a hundred kb centromeric of the DR-genes lies the DQA1 and DQB1 locus which encode the α- and β-chain of the DQ-molecule respectively. Further 500 kb centromeric of DRB genes lies the genes for the DP-molecule, DPA1 and DPB1. In-between these the DN and DO molecules are encoded (Fig. 2).

A few other genes with sequence similarities to the expressed HLA class II genes are also found in this region. These are DQA2, DQB2 and DQB3 genes. They might be a result of duplication of the class II genes and they are not known to be expressed. They could potentially serve as a sequence pool from which creation of new alleles can occur.

Both the α- and β-chain encoding genes have similar structures, with six exons corresponding to the signal sequence, the two extracellular domains, the transmembrane region, the cytoplasmatic tail and the 3' untranslated region. Most of the polymorphism lies in the

second exon, which encodes for the peptide binding part and TcR interacting part of the HLA class II molecule[13].

The polymorphism of the expressed HLA class II genes is as great as for the class I genes, with e.g. DRB1 alleles reported in June 1997 (Table 1)[12]. Only one gene, the DRA, express only limited polymorphism (only two alleles known).

Other genes with immunological function in this region are the HLA-DMA1, DMB1, TAP1, TAP2, LMP2 and LMP7 genes, all encoding proteins important in peptide transport and presentation and also expressing limited polymorphism[14].

HLA class III

The class III region is a 1100 kb long part of the chromosome between the class I and class II region. Genes for complement, cytokines, heat shock proteins and glycocorticoid biosynthesis are encoded in this region, as well as a large number of genes with as yet uncharacterised function and expression[14].

Table 1. Number of alleles for the different HLA genes known in April 1997.

	Molecular characteristics	number of alleles
HLA-A	Class I α-chain	90
HLA-B	Class I α-chain	188
HLA-C	Class I α-chain	48
HLA-E	associated with class I 6.2-kB Hind III fragment	5
HLA-F	associated with class I 5.4-kB Hind III fragment	
HLA-G	associated with class I 6.0-kB Hind III fragment	8
HLA-DRA	DR α-chain	2
HLA-DRB1	DR β-chain determining specificities DR1, DR2, DR3 etc	190
HLA-DRB2	pseudogene with Drβ-like sequences	
HLA-DRB3	DR β-chain determining DR52	14
HLA-DRB4	DR β-chain determining DR53	9
HLA-DRB5	DR β-chain determining DR51	12
HLA-DRB6	DRB speudogene found on DR1, DR2 and DR10 hapoltypes	3
HLA-DRB7	DRB speudogene found on DR4, DR7 and DR9 haplotypes	2
HLA-DRB8	DRB speudogene found on DR4, DR7 and DR9 haplotypes	
HLA-DQA1	DQ α-chain	19
HLA-DQB1	DQ β-chain	32
HLA-DQA2	DQ α-chain related sequence, not known to be expressed	
HLA-DQB2	DQ β-chain related sequence, not known to be expressed	
JLA-DQB3	DQ β-chain related sequence, not known to be expressed	
HLA-DOB	DO β-chain	
HLA-DMA	DM α-chain	4
HLA-DMB	DM β-chain	5
HLA-DNA	DN α-chain	
HLA-DPA1	DP α-chain	10
HLA-DPB1	DP β-chain	82
HLA-DPA2	DP α-chain-related pseudogene	
HLA-DPB2	DP β-chain-related pseudogene	
TAP1	ATP binding cassette transporter	5
TAP2	ATP binding cassette transporter	4
LMP2	Proteasome-related sequence	
LMP7	Proteasome-related sequence	

HLA nomenclature

The nomenclature of the HLA system is complicated indeed, mostly due to the fact that different methods have been used over the years to investigate these genes and molecules. One of the earliest methods used was cellular assays, in which cells with different HLA haplotypes (combinations of alleles from different locus) were mixed in culture. T cells from an HLA incompatible individual will react against cells expressing another HLA type (an allotype). Cells could thus be sorted into groups that tolerated each other and those that responded to different allotypes. These different specificities were given names like Dw2. Essentially, this is a functional test of all HLA molecules on the cell surface.

Further characterisation of HLA class II was achieved by using antibodies in serologic typing, a method that was used from the beginning for HLA class I characterisation. It was now possible to identify the individual molecules on the cell surface as e.g. DR and DQ. Dw2 positive cells could now be assigned as carrying a DR2 and a DQ1 molecule, termed DR2,DQ1 as haplotype. Later the DR2 specificiteis was found to carry more variations and could be split into DR15 and DR16. Similary, the DQ1 specificities was split into DQ5 and DQ6. But antibody specificites are limited to variation accessible to them and can not detect variations that e.g. are hidden in the peptide binding groove, except maybe indirectly as peptides presented by other HLA molecules. Such variations can however be detected by analysing the nucleotide sequence of the HLA genes. Genomic methods used have been e.g. restriction fragment length polymorphism (RFLP) and more recently the PCR based methods. The nomenclature was now changed to consist of the gene name followed by a star and then an allele number of four digits

written in italics, where the first two dig-its gives the corresponding serological specificity and the two last digits give the allele designation The variants of alleles that were detected as DR15 with serology have now been termed *DRB1*1501* to *DRB1*1506*[48].

HLA polymorphism

The HLA genes are the most polymorphic genes known in the human genome. No particular allele dominates in frequency and the distribution of alleles vary between different populations. Most variation is found in Africa[49] from which presumably smaller groups of people have migrated to Europe and Asia. Most of the variation between alleles is not due to new single base mutations, but rather a shuffling of the same sequence motifs into different combinations, created by crossing over and gene conversion. The heterozygosity of the HLA genes is high, indicating that there is a selective advantage for the immune system to have variation within these genes. Similar variations exist in MHC of other animals and the similarity between different species shows that allele diversification started before the origin of species.

HLA haplotypes

If all alleles of the HLA genes where to be combined randomly, over 10^{10} combinations could be generated. However, HLA genes encoded closly together on the chromosome are inherited together as haplotypes. The tight linkage diequilibrium between the DR and DQ genes ensures that, at least in a specific population, one *DRB1* allele is mostly, if not always, inherited with the same *DQA1* and *DQB1* alleles (Table 2).

Table 2. Common HLA class II haplotypes and their allele frequencies in the Swedish population.

HLA-DR serological	**DR subtypes genomic**	**broad specificities**	**DQA1**	**DQB1**	**phenotype frequency (n=102)**	**gene frequency (n=204)**
DR1	*0101-02		*0101	*0501	25%	14%
DR103	*0103		*0501	*0301	1%	0%
DR15	*1501-06	DRB5 (51)	*0102/3	*0601/2, *0502	26%	15%
	*1501	DRB5 (51)	*0102	*0602	26%	15%
DR16	*1601-08	DRB5 (51)	*0102, *0501	*0301/*0502	3%	1%
	*1601	DRB5 (51)	*0102	*0502	3%	1%
DR17	*0301, 0304-05	DRB3 (52)	*0501	*0201	22%	12%
DR18	*0302-03,06-11	DRB3 (52)	*0401	*0402	0%	0%
DR4	*0401-24	DRB4 (53)	*0301/2	*03, *0401/2	36%	20%
DR11	*1101-30	DRB3 (52)	*0501,*0102/3	*0301,*0501,*0603,*0201	11%	5%
DR12	*1201-05	DRB3 (52)	*0104,*0501	*0501,*0605,*0301	7%	3%
DR13	*1301-30	DRB3 (52)	*0103/2,*0501	*06,*0301	22%	12%
DR14	*1401-29	DRB3 (52)	*0104,*0501	*0503,*0301	6%	3%
DR7	*0701	DRB4 (53)	*0201	*0201,*0303	15%	7%
DR8	*0801-16		*0401,*06,*01	*0402,*03,*06	10%	5%
DR9	*0901	DRB4 (53)	*0301/2	*0303	2%	1%
DR10	*1001		*0105	*0501	2%	1%

It is unclear if there is any selective advantage to have a special combination of alleles in a haplotype, or if this is merely an effect of the distance on the chromosome, or lack of recombinational hot spots. Studies of the variations of recombination frequency along the chromosome shows that there is no recombination between DR and DQ, but probably several hot spots between DQ and DP.[50, 51] The DQA1 gene product might pair with a DQB1 gene product from another haplotype, making the variants of DQ molecules expressed on the cell surface even greater. It is also possible that special DQA1 and DQB1 alleles are inherited together because the heterodimer they form is selectively advantageous, or at least not disadvantageous.

Even though recombinations have been shown between the HLA-B and HLA-C genes, the close distance on the chromosome will give rise to a strong linkage disequilibrium between B and C alleles[50]

HLA ASSOCIATED DISEASES

Many diseases are associated with the HLA genes, i.e. a special HLA haplotype occurs more frequently among patients compared to the healthy population. These diseases often have autoimmune features, characterised by a immunological response against a specific target tissue. Different diseases are associated with different HLA alleles, e.g. insulin dependent diabetes mellitus (IDDM) with DR4 and/or DR3, Hashimoto´s thyroiditis with DR5, SLE and DR3, rheumatoid arthritis (RA) with DR4, myastenia gravis (MG) with B8, DR3[52]

It is not clear what role the HLA complex has in the etiology and pathogenesis of these diseases. Several possibilities have been put forward. Since the function of HLA is immunological, it seems feasible that it is the HLA molecules themselves that execute their immunological function in the HLA associated diseases, at least in those diseases which have autoimmune characteristics. The two levels at which HLA molecules can influence an immune response are the selection of the T-cell repertoire in the thymus and activation of an immune response by presentation of peptides outside thymus.

Another possibility is that, yet unidentified, disease-causing genes are closely linked to a special HLA haplotype and that they therefore are inherited together. There are many possible and interesting candidate genes that may influence an immune response encoded in close proximity to the HLA genes, e.g. TNF, complement, TAP and DM. Though, to explain an association these genes have to be polymorphic, which have been described for e.g. the DM, TAP, LMP and TNF genes. Some diseases, that

originally were thought to be HLA associated, have turned out to be due to a disease gene encoded in or near the HLA region, e.g. hemochromatos and 21-hydroxylase deficiency. Other diseases, e.g. systemic lupus erythematosus[53], coeliac disease[54] and type 1 diabetes mellitus[55,56], shows indications of that more than one locus within the HLA contributes to susceptibility.

If a single gene is to explain a disease etiology, all or most of the patients with the disease have to carry that mutation of the gene. A few diseases do have a 100% or near HLA association, e.g. anchylosing spondylitis with B27 and narcolepsy with Dw2. However, most of the other HLA associated diseases show only a moderate increase of the HLA haplotype in the patients, distinct from that of the general population. Thus, carrying the associated HLA haplotype is not sufficient to cause the disease, since this haplotype is also found in healthy individuals. Also, carrying the associated haplotype is not necessary to cause the disease, since there are patients with other HLA haplotypes that also have the disease. So these diseases are probably multifactorial and HLA could be one factor of several. Moreover, the diseases might have several genetic components, which is indicated by the difference in concordance rate between monozygotic (MZ) and dizygotic (DZ) twins, where typically the rate for MZ is about five times that of DZ.

Narcolepsy

Narcolepsy is characterised by irresistible sleep attacks, lasting 5 to 30 minutes, during the day. It is also characterised by cataplexy, an abrupt loss of muscle tone, especially when the patient becomes emotionally excited. One serious danger for narcoleptic patients is car driving, where 40% of the patients admitted that they had fallen asleep during driving.

Drugs that improve transmission at monoamine synapses and drugs that inhibits REM sleep helps[57]

There is a strong familial component in narcolepsy. The disease have sometimes been running in the family for several generations and siblings have a 20-40-fold higher risk of developing the disease. This suggests that there is a group of genes that specifically regulates sleep and that disregulation of these genes might lead to narcolepsy. The onset occurs at 15-25 years of age and the disease affects 0.02-0.06% of the population in the United States and Western Europe. A concordance rate of 25-31% between MZ twins suggests that narcolepsy is not a simple genetic disorder and that environmental factors also influence. In Japan, 100% of the narcoleptic patients carry the DR15,DQ6,Dw2 haplotype, whereas the figures for Caucasians is 90-100% and for African-Americans 70%. DQ has been suggested to be the more important part of the haplotype since many of the DRB1*1501 negative patients carry the DQB1*0602 and DQA1*0102 alleles. The strong association to HLA implies that narcolepsy might be an autoimmune disease. But there are also patients that carry neither DRB1*1501 nor DQB1*0602, showing that the HLA alleles are not necessary and, since these alleles is carried by around 30% of the general population, the penetrance of the disease is low[58]**. The etiology is still unknown, but there is a form of narcolepsy that occurs naturally in dogs and where a single autosomal ressecive trait with full penetrance has been identified. Gene isolation is ongoing both in dogs and of the corresponding human gene region**[59]

Multiple sclerosis

Multiple sclerosis (MS) is a chronic neurological disease with an incidence of 2-4/100 000 and a life time risk of 1/500 in north-western Europe. The prevalence is 20-100/100 000. It usually affects people at

the ages of 20 to 40 and is thereby the most common neurological disease of young adults[60]. About two-thirds of the MS patients are women.

MS is characterised by inflammation and formation of well-demarked plaques in the white myelin rich matter of the central nervous system. The symptoms are varied, but could include paresis, impaired vision, paresthesia, ataxia, fatigue and pain. Magnetic resonanse image (MRI) of the brain and detection of oligoclonal bands in the spinal fluid are helpful tools for diagnosing the disease. There are at least two different disease courses in MS, relapsing/remitting (R/R) and primarily chronic progressive (PCP).

MS seems to be due to an immune reaction with myelin as the primary target, whereas the axons are less evidently damaged[61]. Intrathecal humoral as well as cellular abnormalities have been noted in MS. Infiltration of lymphocytes, mainly CD4-positive T cells, and macrophages can be seen[62]. These macrophages and microglia are activated and have an increased expression of HLA class II. Higher levels of cytokines, mainly of Th1 type, and intrathecal production of antibodies are found in the lesions[63, 64]. The myelin seems to be stripped off the axons cells by macrophages, probably directed by myelin specific antibodies that opsonize the myelin[65]. Damage of the myelin might also be directed by a cytotoxic effect of T cells, possibly also by CD4-positive T cells[66]. This might not be by direct interaction with the oligodendrocytes, since these cells do not express HLA class II[67]. Complement has also been suggested as a possible tissue destroying factor, but since the oligodendrocytes express CD59, they are possibly protected against direct complement

attack, although opsonizing by complement can enhance macrophage mediated destruction[68].

There is no spontaneus animal model for MS. A similar disease, experimental autoimmune encephaolomyelitis (EAE), can be introduced in animals by injecting myelin, myelin-derived proteins or peptides from these proteins together with adjuvants, but also in some strains even without adjuvants. The disease can be primed by transfer of activated myelin-specific T cells. The mechanisms of disease etiology delineated from EAE indicates that MS is an autoimmune disease[51,64,69]. This is also indicated by the finding of autoreactive T and B cells directed against myelin, that are found in MS patients.

Even though the etiology of MS is unknown, it is certain that genes can influence the susceptibility and that environmental factors also are needed, though the relative importance of these factors is not clear[70,71]. The comparably low concordance rate of about 4% between full-sibs and of 35% between MZ twins[71-73], emphasises that the genes conferring susceptibility are not sufficient for development of the disease. The importance of an environmental factor is also supported by the gradient of the incidence seen at both the Northern and Southern hemisphere[71]. The increased incidence of MS in the Faeroe Islands during the second world war is also an indication, though this studies have been questioned lately as being more dependent on, among other things, the presence of neurologists[74].

One argument against the importance of the environment is the adoptee study of Ebers, which shows that the concordance between adopted siblings is not higher than the background prevalence in the population, even though the environment is shared[71]. However, this could

also mean that the environmental factor is common enough to expose the vast majority of the population, in which case susceptibility genes greatly influences the risk, but where the environmental factor still is essential.

Viruses have long been thought to be the actual trigger of MS, where an exposure during childhood and additional events before disease onset has been suggested. However, MS is not likely to be an effect of direct infection, since no specific virus has repeatedly been isolated from MS lesions. MS possible resembles e.g. post-infectious encephalomyelitis more, in that other mechanisms than the direct elimination of the infection leads to a type of autoimmunity. The autoimmune reaction may then prevail long after the virus have been cleared from the body.

The now established genetic predisposition for MS was first suggested by the familial aggregation of the disease shown as an increasing prevalence with increasing degree of kinship[76]. It is also indicated by the difference in prevalence in different ethnical groups, even when these are living in the same area, e.g. gypsies in Hungary and Lapps in Norway[77]. This could of course also be due to an increased sharing of environment. Comparison of the difference between the concordance rate in MZ twins (35%) and DZ twins (4%) is the strongest evidence that genetic factors are involved in the etiology of MS and, moreover, that this most probably involves several genes. Many efforts have been made to identify these genes and contradictory results have been reported from different studies of e.g. the T-cell receptor, immunoglobulins and the myelin basic protein. The only genetic factor which repeatedly has been verified in MS is the HLA genes.

HLA association in MS

Population based association studies shows weather or not a gene marker is increased among patients compared to controls. In 1973, Jersild et al. identified the class II specificity Dw2 as associated with MS[78]. This has since then been verified in many studies and, with new HLA typing techniques, with increasingly higher resolution[79-82]. Earlier studies found association to A3 and B7, but these associations were later regarded as secondary to class II associations once these were established.

After a few misunderstandings during the serological era, the Dw2 has been shows to be "The MS haplotype" world-wide. The Japanese and Arabian association to DR4 and DR6 were not confirmed[83, 84] and indeed an increase of Dw2 was noted in MS patients, even though the haplotype is less common in these populations. The incidence of MS is typically higher in countries where the allele frequency of Dw2 is high. One exception is Sardinia where Dw2 has not been shown to be associated with MS, although the prevalence of MS (1/1000) is fairly high. The misunderstanding during the serological era was due to that in the case of Dw2, in contrary of what might be expected, the resolution of the typing with cellular methods was actually higher than the serological techniques. This is exemplified by that the DR2 association in Hungarian gypsies, which have a low incidence of MS, was shown to be of the subtype DRB1*1601, whereas the DR2 association in Scandinavians, which have a high incidence of MS, was shown to be DRB1*1501[85].

HLA linkage in MS

Linkage studies are made in families and gives answer of if a genetic marker is linked to a disease gene, i.e. if these two are inherited together more often than expected. This can be done in sib-pair analysis or in multiplex families (families with more then one case of MS). Eighty per cent of MS cases are sporadic and 20% have at least one other known case in the family. A few studies have found linkage of HLA and disease in MS families[86], but there are also studies that do not (Table 3)[87-90].

Other genes of importance in MS

Other genes with immunological functions within the HLA complex have been investigated for a role in MS. Linkage between MBP and MS was described in a Finish material, but this has not been verified in independent studies[70, 71]. Association between MS and the LMP and DM

Table 3. Studies of linkage between HLA and MS.

Country	no of families studied	no of sibling pairs	results
Canada[87]	36	40	negative
Scotland[88]	34	31	negative
UK[89]	115		negative
Australia[90]	100		positive
Sweden[91]	9	9	positive
Italy[86]	28		positive
Finland[92] 21			positive

Swden (paper V)	49	19	positive

genes have not been established[93], and the results from studies of the TAP2 polymorphism is contradictory[94-97]. Studies of microsatellite markers close to TNF have also failed to show any additional association[98]. Moreover, for some haplotypes there are linkage diequilibrium between the DR,DQ genes and TAP or DM and additional associations found for this markers have to be tested in HLA matched patients and controls[51].

The identification of the a ytotoxic T lymphocyte antigen 4 (CTLA-4) association in diabetes is however promising and preliminary data in MS and other diseases show that this might be a factor of importance in other autoimmune diseases as well.

Large scale genomic screening has been performed in MS. Here, microsatellite markers spanning the total genome are tested for linkage with disease, mostly in sib-pairs but also in whole families[99-105]. Apart from verifying the HLA region as important, these studies found few other gene regions that were confirmed to be of importance in more than one investigation. However, they could exclude 80-90% of the genome as not being important in MS

AIMS OF THIS STUDY

- To develop a PCR based genomic typing technique for typing of the DQA1 locus.
- To characterise the DQA1*0104 carrying haplotypes.
- To characterise a new DRB1 allele.
- With PCR based genomic typing characterise the alleles within the Dw2 haplotype in MS and narcolepsy.
- To investigate linkage between HLA class II and MS in Swedish multiplex families.
- To investigate additional HLA associations, other than Dw2, in the HLA region and to analyse if there are any relationship between class I and class II alleles in MS.

MATERIALS AND METHODS

Polymerase chain reaction (PCR)

In 1993, Kary Mullis was awarded the Nobel price in chemistry for the principles of PCR[193], showing that the same system used in cells for DNA replication could be transferred to the test tube. The requirements is a DNA template, a DNA polymerase enzyme, nucleotides, primers complementary to the starting point at the template plus a variation in temperature to separate and re-anneal the DNA strands. With the polymerase from the *Thermus aquaticus*, which has a optimum working temperature of 80°C, automated temperature cycling could be used and the test could be performed in a few hours.

PCR amplification with sequence-specific primers

In this method we use the principle that a completely matched primer will allow more efficiently amplification in the PCR reaction than one with one or several mismatches. A series of primer pairs are designed to amplify one allele or a group of alleles each, typically designed to anneal with sequence combinations in the 5'- and 3'-end unique for that allele or allele group. Positive reaction is detected as an amplified PCR product of correct length in an ethidiumbromide-stained agarose gel. A sample of genomic DNA, e.g. from a blood sample of an individual, is added to all the different primer pairs that are going to be tested for and the DNA will only be amplified in those reactions where the primer pair is perfectly

matched. From reading the pattern of amplified reactions the HLA alleles can be determined. An internal positive control by primers specific for a non-polymorphic gene, ensures that no specific HLA allele will be missed due to failure of amplification.

Not all primers are suitable for sequence-specific amplification. Different combinations have to be tested in already characterised materials, such as the HLA workshop cell lines, when the method is developed. When only one alternative primer exists, e.g. because there is only one nucleotide difference between two alleles, the primers can be modified to be more specific by making it longer or introduce additional mismatches a few nucleotides from the 3'-end.

All primers will, with enough cycles and low annealing temperature, amplify any DNA sample. The PCR parameters are adjusted to avoid unspecific amplifications. They also have to be standardized so that all PCR reactions can be run in the same cycler. We have adjusted the primers used in our system to be specific in the following cycling parameters:

1. 2 min denaturation
2. 10 cycles of 94°C denaturation in 10 seconds and 65°C annealing plus extension in 60 seconds.
3. 20 cycles of 94°C denaturation, 61°C annealing in 50 seconds and 72°C extension in 30 seconds.

An addition of glycerol in the PCR buffer will also increase the stringency. This is especially true for HLA class I typing, which require a glycerol concentration of 5%-7% compared to class II typing that is less sensitive, accepting a concentration of 0% to 10% for optimal amplification of all primer mixes. To save time a dye, cresol red

(Sigma®), that does not influence the PCR reaction, is added to the PCR mixture before the cycling. This, together with the glycerol, will make adding loading buffer before the sample is run on gel unnecessary.

The main advantage with sequence-specific PCR is that sequence polymorphisms are detected *cis*, uninfluenced by what allele the other chromosome carries. This becomes even more important as new alleles are detected, many which are combinations of two already known sequence motifs and thus can not be distinguished from the already known alleles in heterozygous combinations (Figure 8 in paper I). Another advantage is that the time from setting the reaction until the interpreted result is short, which make the method suitable for routine clinical work and HLA typing on call.

Other genomic HLA typing techniques

Other PCR based typing techniques are available:

- PCR with sequence-specific oligonucleotides (PCR-SSO)[104], where the DNA sample is first amplified by a locus specific primer pair and after amplification the PCR-product is hybridized with sequence specific oligonucleotide probes. This technique takes longer time and does not distinguish between *cis* and *trans* encoded polymorphisms. It is suitable for large screenings for HLA alleles, since many samples can be run at the same time.

- PCR-RFLP, where the amplified PCR-product is cut by restriction enzymes and polymorphism identified by the fragment size of the cut product.

- **Sequenced-based typing[105,106] where the sample is sequenced in an automated DNA cycle sequencer and run on the ABI system with direct readout into computers. Software is used for the interpretation of the sequence and direct assignment of HLA alleles. With this technique you will directly identify new alleles. However, since the alleles from both chromosomes are sequenced at the same time, there are some heterozygous combinations that cannot be distinguished from each other but which have to be elucidated by selective PCR afterwards. Moreover, it is hard to interpret heterozygous individuals for e.g. some DQA1-alleles, where allele differences are due to deletions[107].**

Sequencing

DNA sequencing used to identify new polymorphisms in this study, was the conventional dye dideoxynucleotide cycle sequencing[108]. (ABI)DNA is first amplified with a biotinylated primer pair. The specific PCR product was pulled out with magnetic beads and a second PCR reaction was performed with dye ddNTP's. Both Sequenase and Taq enzyme were tested, were the Sequenase gave more reproduceable results.

MS patients in this study

Both sporadic cases of MS and families with multiple cases of MS has been used in the studies in this thesis. In paper IV, the study of the MS associated haplotype, 55 patients with clinically definitive MS and 45 patients with cataplectic narcoplpsy, all with the HLA-Dw2 phenotype, were investigated. In the 49 families investigated in paper V, 92 individuals had MS (75% women) and 117 were unaffected family members. The patients were diagnosed according to the Poser criteria[109].The sporadic cases were all from the neurology clinic "the MS centre" at Huddinge hospital. The families were reached through ad-

vertisement. In paper VI, 200 MS patients were divided into two groups, where the first group (n=87) was used for identification of new associations that subsequently were tested in the second group (n=113). For analysis of previously noted divergences, both groups were analysed together.

Controls used in this study

Homozygous cell lines, clinical samples as well as donor spleen cells were used to develop the PCR-SSP typing for DQA1. A selection of Caucasian, African and Orientals as well as a selection of DR2 positive MS- and narcolepsy patients and controls were used to verify the haplotypes described. In the linkage study a panel of 250 healthy Swedish controls were used as well as healthy family members from the multiplex families. Two separate groups of controls, 102 blood donors and 108 necro kidney donors, were used for the association study in the last paper.

STATISTICS

Association

Association analysis was done with Fisher's exact test according to the formula of Wolf in the computer software InStat (GraphPad, SanDiego). When new associations were analysed, a subtraction of chromosomes carrying the previously well-established associated alleles was done first. This is to ensure that negative associations will not be overestimated and aditional positive associations not underestimated

The strength of the association is given as odds ratio (OR), which is similar to relative risk, but used for retrospective studies.

Correction for multiple comparisons

In a statistically correct study the calculated p-value should be multiplied by the number of comparisons done in the study (Bonferreni correction). This is a special problem in HLA association studies since so many alleles exist and can be typed for. If the material is typed for all the alleles of all HLA loci, the correction factor would be over 700. Since some alleles go together as haplotypes this leads to an overcorrection. Some investigators have chosen to correct only for the number of alleles that are tested for within one locus, even though several loci are investigated. In study VI we divided the material in two groups, where the second group was tested for only a few alleles found to be different in the first group. The second material were then corrected for only the 10 specificities that we tested.

Linkage

Linkage analyses test whether a disease and a marker segregate independently in pedigrees and it can be performed in e.g. families with more than one case of the disease. Evidence for linkage is usually expressed as a lod-score, which is simply the logarithm of the likelyhood for linkage divided by the likelyhood for non-linkage. Classical linkage analysis takes into consideration several parameters such as penetrance of the disease in presence of a marker, recombination frequency between the marker and the disease marker and the frequency of alleles. Thus, even though lod scores may in fact be calculated by hand, the use of computerized analysis has revolutionized linkage analysis. The classical linkage analysis programs are provided in the Linkage package [111, 112].

RESULTS AND DISCUSSION

Genomic typing of the DQA1 locus (paper I)

Paper I describes the genomic typing of the DQA1 locus using PCR-amplification with sequence-specific primers (PCR-SSP). Since the publishing in 1993, five new DQA1 alleles have been reported: the DQA1*0105, *0303, *0502, *0503 and *0504 alleles[48]. The DQA1 typing has been adjusted to pick up these alleles by increasing the number of primer mixes from 12 to 16. All phenotypically different DQA1 alleles can still be assigned, although a few of all possible homozygous and heterozygous combinations give the same amplification pattern. These can however be delineated from the linkage to specific DRB1 alleles in the Swedish population, or by additional primer pairs.

The PCR parameters have been optimised, as shown under material and methods, to include a long initial denaturation step and then a 10 cycles two-temperature PCR, followed by a 20 cycles 3-temperature PCR. This will increase the specificity in the first 10 cycles and increase the amount of PCR product in the last 20 cycles.

The PCR-SSP method has been developed for typing of all HLA class I and class II loci in low resolution and for specific allele groups also in high resolution. These methods are used in paper VI.

PCR-SSP has the advantage of using the same parameters for typing of all HLA loci, which makes it suitable for routine laboratory use. It also has the potential to detect new allelic variations, since the method does not detect the same variability as in SSO and sequenced based typing have to deal with (Fig. 8 in paper I).

Further improvement of the method might be feasible by real time PCR, were the PCR product is measured during the PCR reaction. The data can then be transferred into the computer and interpreted directly by software for HLA allele assignment. This would eliminate writing and interpretation errors and the gel-loading step would not be necessary. The use of an internal control might be a problem that hopefully can be solved by the use of different chromafores.

In routine use in our transplantation laboratory, both HLA class I and class II typing by PCR-SSP is used today. Unrelated bone marrow donors are typed for A, B, C, DR, DQ in low resolution first and further subtyped when necessary. DP is typed for with high resolution directly. Less analysis is made for organ transplantation. The resolution of HLA typing actually needed before transplantation is not possible to evaluate before stringent, high resolution typing methods are used to type both donors and recipients. Such studies are on their way and a first retrospective typing with PCR-SSP and PCR-SSO of 215 kidney transplants shows that the survival of completely matched kidneys were greater than those that were shown to have incompatibilities[113]. Comparison of class I serology and PCR-SSP shows that up to 10% of the typing done with serology had either missed an allele or failed to distinguish between different allele groups and some were also wrongly assigned (Schaffer, manuscript in preparation). Similar findings of 10-20 % discrepancy between serology and genomic typing techniques have been reported by others[114].

Is it then necessary to type for the minute differences detected by genomic typing techniques? Minute differences of single amino acids have been shown to have effect on transplantation outcome[115,116] and amino acid differences in parts of the HLA molecule that do not have contact with either TcR or the peptide could potentially give rise to an

immune response if they are presented as peptides in the recipients HLA molecules. On the other hand, some degree of mismatch might have an advantageous effect on the recipients leukaemia after bone marrow transplantation (graft-versus-leukaemia effect). A successful outcome with one amino acid difference was shown for a bone marrow transplant[117], but whether the differences in acceptable mismatches is due to the position of the mismatch or other reasons have to be investigated further.

New haplotypes and alleles (paper II and III)

New alleles are reported every month and within the 1997 April update[118] 188 DRB1 and 19 allelic variations of DQA1 were recognised (table). Paper II describes haplotypes expressing the DQA1 allele and paper III describes a new DRB1 allele (DRB1*1415).

The lately discovered DQA1*0105 allele differs from the DQA1*0104 only with one nucleotide in the 4th exon[118]. This exon was not investigated in paper II. DQA1*0105 is carried on the DRB1*1001 haplotype, whereas the DQA1*0104 is carried on the DRB1*1401 haplotype.

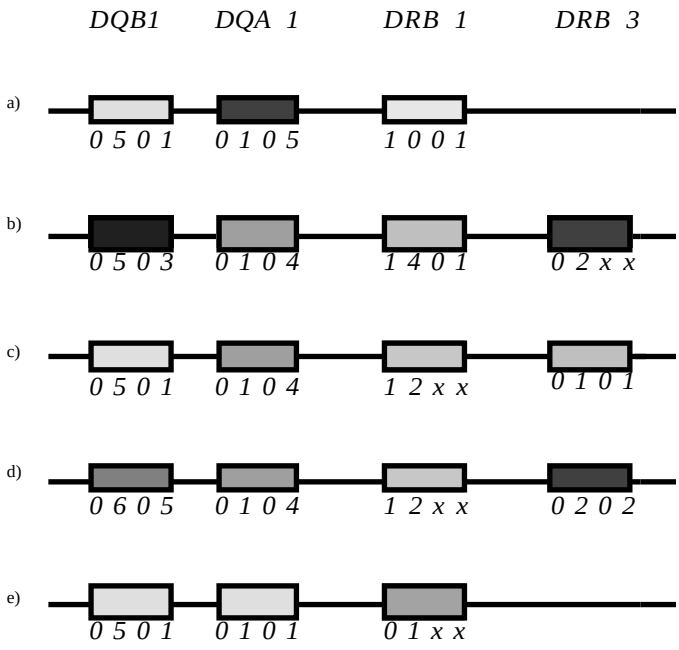

Fig. 4. The haplotypes carrying the DQA1 alleles *0105, *0104 and *0101 and their corre-sponding DQB1, DRB1 and DRB5 alleles.

The DRB1*1415 allele described in paper III did carry the DQA1*0104 allele as well as other DR14 haplotypes that have been found during routine typing. There are however 29 DRB1*14 alleles described today of which many have uncharacterised haplotypes. The allele frequency of the DRB1*14 alleles and the DRB1*1001 allele is 3% and 1% respectively, and presumably the DQA1*0104 and DQA1*0105 are carried in about the same frequencies. Only a few of the DRB1*12 alleles carry the DQA1*0104 in the African individuals tested.

The close similarities between DQA1*0104 and *0105 indicates that the variation was created by point mutation after recombination between the DRB1*14 and DRB1*1001 or DRB1*12 alleles occurred. Interestingly, the DQα**– and** β**-heterodimers formed by the DQA1 and DQB1 alleles of the DRB1*14, *1001 and *12 haplotypes will all be different. Thus, the variability of the DQ heterodimer can by combinations of different alpha and beta chains give rise to a variability comparable with the amount of alleles expressed for the DR heterodimer.**

The persistence of these DQ variations on distinct haplotypes world-wide indicates that such variations might be functionally important for the immune system.

The DQA1*0104 and *0105 differ from the other DQA1 alleles in the first exon, encoding for signal sequence and part of the DQ. They also differ more upstreams in the promoter region, which is not unusual for the DQA1 gene, where different polymorphic promoters can occur together with the same DQA1 allele and vice versa[119]. These differences are more likely to change expression levels than influencing peptide binding or T-cell interactions. There are several examples of alleles that have lost their expression capacity, which are detected by genomic typing but not serology[120], but whether this is true for the DQA1 alleles is not known.

A few new alleles carry newly discovered nucleotide mutations, whereas most new variants are created by reombinations of already existing mutations[121]. Recombinations seems to be common for the DRB1*14, *08, *11, *13 and *03 allele groups, which have large numbers of subtypes reported, compared to e.g. the number of DR1 alleles. Many of the new alleles reported are rare and found in other population groups than the North European, and one can question of the relevance of typing for all these variants when transplantation between e.g. Scandinavian patients and donors are considered. It is important that cell lines are created when a new allele is found to enable update of HLA typing methods. It is also valuable to have continuous update of the allele frequencies in the population.

The DR2 haplotype associated with multiple sclerosis and narcolepsy (paper IV)

Paper IV is an investigation of the alleles within the narcolepsy and MS associated HLA haplotype, DR15,DQ6,Dw2. Two additional DRB1*15 alleles (the DRB1*1505 and *1506) and two additional DRB1*16 alleles (the DRB1*1607 and DRB1*1608) alleles have been reported since 1995. In addition, six new DRB5 alleles (DRB5*01011-0108N, *0202-0204) have been reported. This will not influence the result concluded from paper IV, though we can say that the DRB5*0101 that we typed for acctually is DRB5*01011. The narcolepsy and MS associated haplotype can thus be considered to be DRB1*1501, DRB5*01011,DQA1*0102,DQB1*0602 (=DR15,DQ6,Dw2).

Further study of narcolepsy in other ethnic groups shows that the association to DR15,DQ6 is not always 100%, as in Japanese and our Swedish patients with narcolepsy. It is however hard to draw any conclusions of which part of the HLA class II haplotype that is most important, since the other haplotypes that are carried by the DR15,DQ6,Dw2-negative patients have either other DRB1 alleles or DQ alleles - or both. However, the majority (64%) of the DRB1*1501 negative patients do carry the DQB1*0602 and DQA1*0102 alleles. The differences between different ethnic groups could in part be due to difficulties in diagnosing the disease in different countries. Since narcolepsy does not have any clear autoimmune etiology, a role of a gene in linkage disequilibrium with the associated haplotype is possible. This does not rule out the possibility that such a gene product also interplay with specific alleles of the HLA system.

Higher resolution in HLA typing has shown that the previously cellularly and serologically defined MS associated alleles only consists of one of the several possible subtypes in both patients and controls. One exception in our MS patients and controls is the DQ6 allele, which was typed as DQB1*0602 in the majority of the cases, but also as DQB1*0603

in 2% of the patients and 2% of the controls. The difference between the *0602 and the *0603 allele is however minute and might not have any influence in the etiology of MS[120]. DQB1*0603 is expressed on other haplotypes not associated with MS as well (DRB1*13), which, if anything, would mean that the DQB1*0603 is not disease promoting by itself. To continuously have the same haplotype within a population, even as detected with higher resolution, indicates that the disease promoting effect lies within the whole haplotype and that this haplotype probably spring from a common ancestor. All genes within that haplotype are thus of potential interest to study. Efforts have been made to identify epitopes within the HLA molecules that might show increased associations to MS[80,123], in line with that has been identified for coeliac disease, IDDM (DQB1 position 57)[45-47,126] and humoral immunodeficiencies[127]. These suggestions have not been verified in independent studies[84].

Linkage in MS (paper V)

Twenty percent of MS cases are familial, i.e. the patient has one or several relatives that also have MS. This familial aggregation is also illustrated as an increased risk for first degree relatives to acquire the disease, as well as the even higher concordance rate for monozygotic twins. However, disease within families does not point to strictly genetically influence, since families also share environment. To establish the role of one gene within families, this gene has to be inherited more often than expected together with the disease. The statistical measure of the evidence for linkage is classically calculated as lod scores. A lod score of 3, or the odds 1000:1 that a genetic marker is linked with the disease, is regarded as significant at the conventional p=0.05 threshold. We calculated the lod score for HLA-DRB1, -DQA1 and -DQB1 in 49 families with at least two patients with MS and found a

significant lod score for DRB1 (=3.03) for a dominant inheritance model. Intermediate and recessive model of inheritance were also tested, but this gave lower lod score values, as did the DQA1 and DQB1 loci. Moreover, the highest lod score was obtained for very low penetrance (3%), which is in line with the association studies, showing that most individuals (30% of the population) that carry the associated DR15,DQ6,Dw2 haplotype do not develop MS. The dominant inheritance linked to the DR2 haplotype have been shown before, however this is not confirmed by all studies and the mode of inheritance can not be said to be completely defined yet [70, 84-139].

How, then, can a gene be both dominant and have low penetrance? Since MS probably is a multifactorial disease, additional factors are needed to develop the disease. It is possible that the risk of developing disease increases with the amount of susceptibility genes that the individual carries. The low penetrance could be explained if the environmental factors are uncommon and that many people do have genetically high risk of developing MS, but never come in contact with the triggering and disease promoting factors required. An alternative explanation is that the environmental factors are common, but these interact with several different genes in a multistep procedure that in the end leads to the development of MS. Some of the events, like rearrangements of the autoreactive T and B cells receptors, are stochastic and the chance of rearranging an autoreactive receptor might be small. Moreover, these specific T cells have to survive the selection in thymus, an event that is dependent on HLA.

Highest lod score value was obtained with a recombination frequency of zero. That is, either we are looking at the actual disease connected gene, or the disease causing gene lies so close to the HLA class II genes that recombinations are extremely rare. This is also in line

with the data from the association studies were flanking markers of the HLA class II region have failed to show association that is independent of the DR15,DQ6,Dw2 association.

Although standard lod score analysis is tremendously powerful in identifying linkage within 20-Mb gene segments (the HLA region is 4 Mb), there are some drawbacks that have to be considered. The method is model-dependent and thus factors like mode of inheritance, allele frequencies and penetrance have to be specified. The allele frequencies of the HLA system is well known since this have been extensively investigated in many populations. The fact that many healthy individuals carry the same associated DR15,DQ6,Dw2 haplotype, tells us that the penetrance is low. The mode of inheritance is however harder to determine, since different studies give different results. This can generate spurious linkage in imperfectly Mendelian conditions like MS. Another disadvantage with the lod score analysis is that where no recombinations occur might be a fairly large gene region, which leaves us with a region of interest, but still not the actual gene. Moreover, if the disease is heterogeneous, i.e. depending on several different loci, a linkage is hard to establish, even though the disease would be caused by clear disease causing mutations. However, analysis of the associated DR15,DQ6 haplotype shows that this is the normal (i.e. not mutated) HLA haplotype carried by many healthy individuals. Thus, the susceptibility-increasing ability of the HLA genes might be a coincidence of normal variation. Since the variation of the HLA system is so extreme, this must be of great advantage for the immune system in defending the body against invading microbes, which might also give the drawbacks that some individuals develop autoimmune disease. These diseases typically develop after reproductive age and such disease susceptibility genes will therefore not select away. Final drawback of the lod score analysis, as

many other genetical analysis, is the vulnerability to errors like typing mistakes, clinical misdiagnosis and locus heterogeneity.

There are other model-free linkage analyses that may circumvent the above mentioned drawbacks. These methods only look at the affected individuals and search for genes that are shared between them. Pairs of sibs are expected to share 0, 1 or 2 parental haplotypes with the frequency of 1/4, 1/2 and 1/4 respectively. Distinction of alleles that are identical by descent (IBD) and alleles that are identical by state (IBS) have to be made, especially since many of the HLA alleles occur in high allele frequency. This requires that the alleles of the parents can be deduced and that none of them are homozygous for that allele. We identified sib-pair in our MS families and found 16 that were concordant. True IBD could not be determined in these few cases, since some of the parents and the siblings were homozygous. Since most of these sibs carried the DR15 haplotype, which occurred in doubled frequency in these families compared to the general population, it is likely that some of the sibs only were identical by state and not by descent. However, the proportion of alleles shared between the sibling differed from that expected on the basis of independent assortment (χ^2_2, $p<0.01$), which support the existance of linkage between the HLA complex and MS (Table 4).

Table 4. Proportion of 16 siblings concordant for MS sharing 0, 1 or 2 HLA-DR alleles.

	0 alleles identical	1 alleles identical	2 alleles identical
Observed	0	6	10
Expected	4	8	4

$\chi^2 = 13.5$ ($p<0.01$, df=2)

To determine if the associated DR15,DQ6,Dw2 haplotype was inherited more often than expected, the MS patients were compared to their healthy sibs (paper V, table 3). This was indeed the case, giving additional evidence of the importance of HLA as genetical predisposing genes.

We have recently performed additional statistical analyses of the 49 multiplex families to test the significance of our findings. This is important since maximum two-point lod scores may in fact be inflated if the model giving the highest lod score is not correct. However, the non-parametric linkage analysis (NPL) of the Genehunter package [128] **showed a clearly significant p-value of 0.0096. In the one parametric model that we tested, also using the Genehunter package, the lod score was 2.8 using a penetrance level of 0.1, i.e. similar to the 2-point score of 3.04. Finally, the affected pedigree member analysis (APM), another nonparametric analysis based only on the affected individuals of the pedigrees, gave a p-value of 0.0056. Thus, we conclude that the statistical evidence for a significant role of the HLA class II genes in MS in the Swedish population is solid.**

Association of DR15,DQ6,Dw2 within the families were also determined. Compared to healthy Swedish controls, the within family DR15,DQ6,Dw2 carriers had a risk of 5.9. Sporadic DR15 positive cases of MS usually have a risk around 3.5. The higher within-family risk could indicate that additional genes within these families contributes to disease susceptibility. If the affected family members were compared to nonaffected, still DR15,DQ6 carriers had an increased risk of 2.8,

indicating that HLA is at least one of the more important susceptibility genes.

Other studies have shown no linkage to HLA and interpreted this as a minimal role of the HLA genes[87,89,92]. The discrepancy between these studies and ours could be due to differences in study design or that we investigate different populations. However, lately a re-appreciation of HLA as important susceptibility gene have arisen. Mostly because of the findings in the large genome screening studies done, where HLA came out as the only genetic factor that was found in all populations studied[99-102]. Moreover, the discrepancy of the linkage studies might only to be expected for multifactorial and polygenic diseases like MS.

A question that remains unsolved is whether the linkage and association found is due to an effect of normal immunological function of the HLA molecules themselves or to a disease causing gene encoded in linkage disequilibrium within the HLA gene region. In our multiplex family study we find that even families without the associated DR15,DQ6,Dw2 haplotype contribute positively to the lod score. This speaks in favour for the HLA molecules themselves, since a mutation of a closely encoded gene would follow only the haplotype on which it first occurred. Having the associated haplotype will increase the possibility that an unlucky immunological event leading to autoimmunity will take place, though this reaction also occurs in individuals with other HLA alleles, but to a lesser extent. Alternatively, the same disease could be caused by several different mutations of a closely encoded gene.

<u>Association with other alleles in the HLA class I and class II region (paper VI)</u>

In this study we have performed a thorough HLA typing by PCR-SSP of the expressed class I and class II genes, i.e. A, B, C, DR, DQ and DP. The aim was to investigate if any other alleles in the HLA system, than the associated DR15,DQ6, could influence the susceptibility to MS. Indeed, both negatively and positively associated alleles were found. These effects were most clearly seen when the combination of class I and class II haplotypes were considered. Specifically, the HLA-A3 allele increased the risk whereas HLA-A2 decreased the risk. In contrary to what has been found before the HLA-A2 and HLA-A3 associations were independent from the DR15 association. B7, however, followed the DR15 haplotype. Carrying the A3 together with DR15,B7 will increase the risk of MS even more then carrying the DR15,B7 with another A allele. Similar, A2 will decrease the risk even for DR15,B7 carriers, i.e. the influence from the HLA-A locus can modify the established DR15,DQ6,Dw2 susceptibility. In contrary to what we have found before[139], there is actually a difference between the HLA haplotype, or at least combinations of alleles, carried by patients compared to controls if this haplotype is compared all the way from the DP genes to the HLA-A gene. Here, class I can have both a disease promoting effect or a protective one depending on allele, whereas only a disease promoting effect was found on the DP side of the DR15 allele. However, from this study we can not say if these alleles lie on the same chromosome or if the effect is due to interaction of the expressed molecules. The analysis of the DQA2 and the C4 genes[84,120], close to the DQ and DR genes, indicates that the haplotype do not extend that far, which means that an interacting effect can be executed in trans as well as in cis, i.e. the effect is due to the HLA molecules themselves and the role they have in immune regulation.

A protective effect both in the MHC class I and class II have been shown in rat models of experimental autoimmune encephalomyelitis

(EAE), induced by a peptide of the myelin basic protein (MBP) in recombinant strain congenic for MHC. The protective effect of the class II genes of the "u" haplotype was mediated by cytokines of a Th2 type of response. These animals developed a immune response to the same MBP peptide as the susceptible rats, but they did not develop EAE. The protective effect found in the class I region was mediated by TGF-β producing CD8-positive T-cells, shown by that the effect could be blocked with anti-CD8 antibodies. The susceptible strains developed a Th1 type of cytokine response. Interestingly, the same MBP peptide was used in all these experiments, which indicates that it is the peptide binding capacity of the MHC molecules that regulates the disease response. The finding that HLA-A2 occurs in less frequency in DR15,DQ6,Dw2 positive patients compared to controls, indicates that similar immunological regulation between alleles of different HLA molecules might also exist in humans. This effect is not completely dominant, since there are patients that have DR15,B7,A2 and still develop the disease.

The animal model shows the effect is due to the MHC molecules themselves, since the rats were congenic for MHC and since they all were injected with the same peptide. If the same is true for humans could be analysed by studying the combinations of the A2, A3, B7, DR15, DQ6 and DPB1*0401 in families. This will indicate if the class I and class II alleles has to be encoded *cis* (which would favour a linked gene hypothesis), or if *trans* encoded alleles sre enough (which would favour a disease regulated effect of the HLA molecules themselves).

Apart from giving rise to different immune responses as in the EAE model, there are other ways in which HLA class I alleles might exert their disease regulating ability. One is the selection of the T-cell repertoire, where the class I molecules either could select or delete

potentially protective CD8-positive T cells. If viruses are the triggering environmental event needed for development of MS, one can speculate that the immune response against the viruses are differently regulated depending on HLA class I alleles. A HLA class I alleles which efficiently presents intracellular peptides from viruses, might lead to a better clearing of the infection at an early stage. This might stop the virus from becoming latent, stop the virus to come out of its latent state, or regulate other ways that possibly could turn a virus infection to a autoimmune reaction.

CONCLUSIONS

HLA AND MS

The linkage and associations shown in this thesis indicate that HLA genes are of importance in MS, but we can still not prove if it is the molecules themselves that are responsible for the susceptibility. However the independent class I association as well as a negative association in the class I region indicate that the HLA molecules themselves are active in susceptibility. If it thereby can be concluded that MS is indeed an autoimmune disease where components of the immune system together with environmental factors cause myelin destruction, there are at least three ways in which the disease mechanism can be elucidated from here:

Hypothesis 1. The HLA association is a thymic effect. The HLA molecules act as selectors of the T-cell repertoire, where some HLA molecules may be more prone to let myelin-directed T cells out from the thymus. Individuals carrying that specific HLA haplotype would then have an increased risk of developing MS. Similarly, there might be combinations of HLA alleles that efficiently deplete such T cells and protect the individual against MS. A protective effect could also be mediated by an increased ability to select disease down-regulating CD8-positive T cells. Myelin-directed T cells from MS patients would then be of interest to study. This has been done many times already, showing that such a T cells are present, but also in healthy controls, although MS patients tend to have more of them. Moreover, the myelin proteins they directed against are not the same in all patients. To further elucidate the mechanisms of the reaction, the T cells of early lesions have to be characterised for antigen specificity, HLA restriction, T-cell receptor rearrangement, cytokine secretion and activation state. Such studies are probably very difficult to perform.

What speaks against this hypothesis is that the binding of specific peptides in thymus does not regulate the T-cell repertoire completely. Transgenic mice expressing only one peptide in thymus still had a large number of different T cells, also showing that the activating peptides need not be related in TcR contact residues to the selecting peptide in thymus[132]. Moreover, studies of T-cell repertoires in HLA-identical and non-identical individuals in families shows that, although the shaping of the T-cell repertoire is dependent on the HLA genes, other non-HLA genes does also seem to be of importance[133, 134].

Hypothesis 2. The HLA association is due to the ability of the HLA molecules to activate myelin-specific T cells. One way of studying this

would be to look at the actual myelin epitopes that are presented by susceptibility associated, as well as protective, HLA haplotypes. It is then essential to look at all the molecules and not only DR and DQ. However, due to the possible heterogenicity of the disease an identification of single autoantigen-specific T cell response in all MS patients is unlikely[66]. Since T cells specific for MBP are found both in diseased and healthy individuals, the presence of these seems to correlate more with genetic background then association with disease. On the other hand, in a twin study of the restriction of MBP specific T cell, a preferential restriction to the DR4 molecule in a DR4/DR13 heterozygot was noted, but a restriction to the DR15 molecules was seen in a DR15/DR4 heterozygot[135]. The different ability of HLA alleles to bind myelin peptides could possibly influence disease outcome and any study of T cells in MS has to be correlated to genetic background. If one finds specific myelin eptiopes and corresponding disease related T cells in MS patients one would have to explain why and how these epitopes comes out from the brain and activates the T cells in MS patients but not in healthy individuals.

Hypothesises 3. MS is a virus-induced disease and the HLA association is due to the ability of the immune system to respond to a virus infection. One hypothesis of how viral infections can lead to autoimmunity is that they cross-react with myelin epitopes. Sequences of mapped HLA bound myelin epitopes in an MS patient could then be searched for in viral genomes. However, it is also possible that viruses have the ability to induce autoimmunity by carrying host proteins with them, for example by incorporate them in the envelope[136]. A screen of viral genomes would then be meaningless. Instead it would be more interesting to look at which viruses that actually have the capacity to infect oligodendrocytes

and then study if they can transfer myelin proteins out from the brain and activate T cells. A relationship to the HLA association is harder to establish in this case, but could be due to differential regulation of the immune response against that virus. Any gene product that possibly interacts with the virus at any stage from infection, latency establishment and reactivation, would be of interest to study. This theory fits well with that MS might be a heterogeneous disease. Apart from that several genes are involved, these genes have to interact with the environmental factors necessary for disease triggering, factors that might be different in different patients and many different viruses might have the same effect. This is also supported by the different pathologies found in MS brains.

In summary, MS might be regarded as a symptom of myelin destruction that can occur in different ways and that might have different etiology. This, together with the notion that MS might not be caused by a single environmental agent, adds to the complexity of the disease. To give us ideas of disease mechanisms in MS, it is of interest to look at genetic factors in relationship to environmental factors and immunological stochastic events, as well as to correlate them to pathological characteristics. In future, such genetical analysis can help us to adjust therapy and predict disease outcome and to identify individuals with high risk for prevention treatments (Fig. 5).

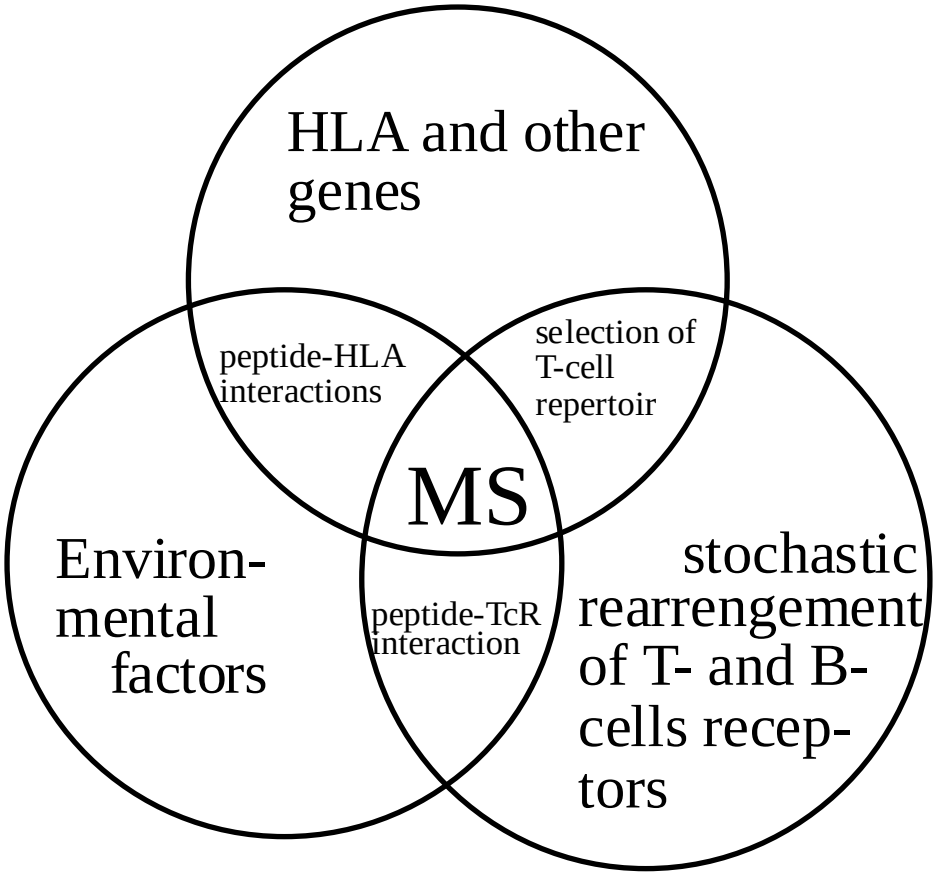

Fig. 5. The relationship between possible events contributing to the development of MS.

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