MARI LUOMALA

Immune System Genes and Multiple Sclerosis

A Case-control Study in a Finnish Population

ACADEMIC DISSERTATION
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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications, that are referred to in the text by the Roman numerals I - VI. In addition, some unpublished data are presented.


VI Luomala M, Lehtimäki T, Huhtala H, Ukkonen M, Koivula T, Hurme M and Elovaara I (2002): The functional polymorphism of IL-10 at position -1082 may be linked to the severity of MS. (submitted)
ABBREVIATIONS

ApoE  apolipoprotein E
APC   antigen presenting cell
BBB   blood brain barrier
Bp    base pair
CCR5  chemokine receptor-5
CNS   central nervous system
CSF   cerebrospinal fluid
CTLA-4 cytotoxic T lymphocyte antigen-4
CXCR3 CXC chemokine receptor-3
DNA   deoxyribonucleic acid
EAE   experimental autoimmune encephalomyelitis
EBV   Epstein Barr virus
HLA   human leukocyte antigen
ICAM-1 intercellular adhesion molecule-1
IL-1β interleukin-1β
IL-2   interleukin-2
IL-4   interleukin-4
IL-10  interleukin-10
IL-1RA interleukin-1 receptor antagonist
INF-γ interferon-γ
MBP   myelin basic protein
MOG   myelin oligodendrocyte glycoprotein
MRI   magnetic resonance imaging
mRNA  messenger ribonucleic acid
MS    multiple sclerosis
PAI-1  plasminogen activator inhibitor-1
PCR   polymerase chain reaction
PPMS  primary progressive multiple sclerosis
RRMS  relapsing-remitting multiple sclerosis
TCR-α T-cell receptor α chain
TCR-β T-cell receptor β chain
Th1   T helper 1
Th2   T helper 2
TNF-α Tumor necrosis factor-α
VCAM-1 vascular cell adhesion molecule-1
VNTR  variable number of tandem repeats

In addition, the standard one-letter abbreviations of nucleotides are used.
INTRODUCTION

Multiple sclerosis (MS) is a chronic, inflammatory and demyelinating disease of the central nervous system (CNS). After trauma it is the most common cause of neurological deficits in young adults. Typically the disease follows relapsing-remitting course progressing chronically over time. The disease course in any individual is unpredictable; it may cause severe disability and present therapies have only a modest effect on it. The etiology and pathogenesis of the disease remain unclear, but immune-mediated mechanisms seem to be central in its initiation and progression. Twin, family and population studies have shown that both genetic and environmental factors affect susceptibility to MS. The HLA DR2 haplotype predisposes to it in Caucasians. Otherwise, the genetic factors involved in MS remain obscure.

The purpose of the present study was to assess the impact of several immune system genes on MS susceptibility and severity in a genetically relatively homogeneous Finnish population.
REVIEW OF THE LITERATURE

1. Clinical features of MS

**Epidemiology.** MS is a chronic neurological disease of the CNS characterized by multifocal inflammatory and demyelinating lesions in brain and spinal cord. About five per 100 000 Finns develop MS every year, although higher numbers have also been reported in Seinäjoki (Sumelahti et al. 2000). It is of note that the incidence of primary progressive MS (PPMS) is remarkably high in Seinäjoki (Sumelahti et al. 2002). The prevalence of MS per 100 000 Finns is about 100 in Uusimaa and in Vaasa (Sumelahti et al. 2001), as against some 200 per 100 000 Finns in Seinäjoki, which is among the highest reported (Sumelahti et al. 2001). Disease onset is most common in young adults at the age of 20 - 30 years, and the disease is about 1.5 - 2 times more common in women than men. A female prevalence is commonly seen in many diseases with autoimmune etiology. The modulatory effect of sex steroids on immune function is proposed as an important primary mediator of the sex difference (Whitacre 2001).

**Clinical features.** MS is clinically extremely variable, showing marked individuality of symptoms. The symptoms and signs of MS reflect the anatomical sites affected by impaired saltatory conduction. Any part of the CNS can be involved, however; cerebrum, optic nerve, brain stem and spinal cord are frequently affected. Common symptoms are muscle weakness, sensory disturbances, fatigue, blurred vision consistent with optic neuritis, inconsistency, pain, cognitive impairment, depression and emotional lability. Typically the disease follows a fluctuating course, with relapses and remissions (relapsing-remitting MS; RRMS). Key triggers of relapses are upper respiratory infections and gastrointestinal infections (Panitch 1994). In most patients with RRMS (80 %), the disease course changes to chronically progressive, when the disease has lasted about 10 - 15 years (secondary-progressive MS; SPMS). About 20 % of affected patients have chronic progressive disease from disease onset (primary progressive MS; PPMS) which may lead to permanent disability within a few years and is equally common in men and women.

There are not good measures to evaluate all aspects of the progression of MS disease. One of the most commonly used is the Kurtzke Expanded Disability Status Scale (EDSS) (Kurtzke 1983). The EDSS has 20 steps beyond 0 (normal), extending to status 10 (death due to MS). EDSS 1,0-3,5 reflects good ability to act, EDSS 4,0-7,0 variable deficts in the ability to walk, EDSS 7,5-8,5 effective use of arms but the need for a wheelchair and EDSS 9,0-9,5 bed-ridden patients who cannot perform self-care functions such as feeding. In one large cohort study on the natural course of MS the time from the onset of MS to the EDSS 4, 6 and 7 were 11, 23 and 33 years, respectively.
(Confavreux et al. 2000). However, one handicap of EDSS in reflecting MS-associated disability is that it is mainly based on the walking ability. Now it is clear that also other aspects such as cognitive capacity should be taken into account in the assessment of functional capabilities of the patient.

**Diagnosis** of MS is based on evidence for at least two attacks, affecting more than one anatomical site (Table 1) (Poser et al. 1983). Magnetic resonance imaging (MRI) is used to visualize demyelination areas in the CNS and enables evaluation of lesions disseminated by anatomical site and time. Recently introduced diagnostic criteria have incorporated MRI and the second lesion can be documented by imaging and need not necessarily be clinically expressed (McDonald et al. 2001). Findings in cerebrospinal fluid (CSF) of oligoclonal bands and increased production of immunoglobulin G (IgG) are not specific for MS, but indicate immunological activity. Measurement of evoked potentials can demonstrate slowed action potential conduction, which is consistent with demyelination.

**Table 1.** Diagnostic criteria for MS according to Poser.

<table>
<thead>
<tr>
<th>Diagnostic classification</th>
<th>Attacks (No)</th>
<th>Clinical evidence</th>
<th>Paraclinical evidence</th>
<th>CSF OB/IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinically definite</td>
<td>2</td>
<td>2</td>
<td>and 1</td>
<td>+</td>
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<tr>
<td>Laboratory-supported</td>
<td>2</td>
<td>1</td>
<td>or 1</td>
<td>+</td>
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<td>definitive</td>
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Clinical evidence = Signs of neurological dysfunction demonstrable by neurological examination
Paraclinical evidence = Lesions demonstrable by means of tests (evoked potential and MRI)
CSF OB/IgG = Oligoclonal banding or increased IgG in the cerebrospinal fluid
2. Pathogenesis of MS

MS is suspected to be a T helper 1 cell-mediated autoimmune disease, but recent studies have suggested a more complicated immunology (see Figure 1 on page 15). A general conception is that environmental and genetic factors can distort immune homeostasis towards harmful sustained immunological responses in the periphery and CNS, leading over time to the clinical symptoms of MS. The primary event underlying the immune responses is not known; autoimmunity, chronic virus infections and neurodegeneration have been proposed in the etiology of immune cell activation in MS (Hemmer et al. 2002)

2.1. Pathology

**Chronic and active lesions of MS.** Lesions of MS are centered on one or several medium-sized vessels and have a tendency to accumulate near the periventricular or outer surfaces of the brain and spinal cord. In chronic lesions myelin sheaths are completely lost; axons are spared and embedded in dense astroglial scar tissue (Lassmann and Vass 1995). The inflammatory infiltrates are mainly composed of lymphocytes and macrophages. Active lesions involve demyelination with little astroglial formation and are infiltrated by numerous inflammatory cells, particular macrophages. As compared to chronic lesions, active lesions have an increased number of B lymphocytes and antibodies (Lassmann and Vass 1995). In addition to chronic and active lesions, chronic active inflammatory lesions and chronic silent lesions can occur in most MS brains.

The pathological hallmark of a focal MS lesion is destruction of the myelin sheath. In addition, axonal injuries occur in most lesions. Myelin damage inhibits the conductance of action potentials, which is considered to be a major cause of neurological symptoms in MS. In demyelination areas, remyelination (Prineas et al. 1993) and axonal repair (Rivera-Quinones et al. 1998) may restore conductance. However, in chronic forms of MS the capacity for remyelination is decreased and permanent functional deficits cumulate, as developing astrogliosis prevents entry of oligodendrocyte progenitor cells into the lesion site. Axonal transection (Trapp et al. 1998) and axonal loss (Bjartmar et al. 2000; Lovas et al. 2000) cause irreversible conduction blockades (Trapp et al. 1998) and would appear to be important reasons for permanent disability in MS (Bjartmar et al. 2000, De Stefano et al. 1998, De Stefano et al. 1999). Axonal loss may occur independent of demyelination (Ferguson et al. 1997, Trapp et al. 1998) even constituting, an early event in the course of the disease (De Stefano et al. 2001), and it also occurs in normal-appearing white matter (De Stefano et al. 1999, Evangelou et al. 2000). The relationship of myelin and axonal damage in MS pathology is unclear, but inflammatory mechanisms obviously contribute to both types.
Heterogeneity of lesions. Biopsies and autopsies from acute MS lesions in 83 cases have suggested that demyelination mechanisms are the same intraindividually but different interindividually. In most cases T-cell- and macrophage-dominated inflammatory reactions were detected, but otherwise lesions showed four different patterns of demyelination (Lucchinetti et al. 2000). In pattern I lesions macrophages were a main contributor whereas in pattern II lesions an important role of antibodies and complement was suggested. In patterns III and IV lesions demyelination was associated with oligodendrocyte death by apoptosis and by non-apoptotic mechanisms, respectively. In addition, it has been suggested that immunological mechanisms could be prominent in RRMS, whereas degenerative events could be a main contributor in the chronic progressive phase of MS (Hemmer et al. 2002).

2.2 Autoimmunity in MS

The ability to distinguish self from non-self is a seminal feature of the immune system. Protective immunity against many pathogens appears to involve the activation of CD4+ T cells and production of effector cytokines. The same would seem to apply in the formation of autoimmune attack.

An overview of the molecules involved in the activation of CD4+ T cells. The human leukocyte antigen (HLA) molecules expressed on antigen-presenting cells (APC) present antigenic peptide to CD4+ T cells, this being fundamental to the initiation of the antigen-specific immune response against pathogens. CD4+ Th cells determine both the specificity and the mechanism of cell-mediated immunity. At molecular level, a trimolecular complex, including HLA on APC, T-cell receptor (TCR) on the T cell and antigenic peptide, is the basis for the specificity of the response.

HLA molecules are heterodimeric, possessing a groove for specific antigenic peptides. HLA molecules are divided into HLA class II (HLA-DR, -DQ, -DP) and HLA class I (HLA-A, -B, -C) molecules. HLA class II molecules are expressed on APCs such as macrophages, B cells, dendritic cells and thymic epitelial cells, and present exogenous antigen to the CD4+ Th cells (Roitt et al. 1996). HLA class I molecules are expressed on most nucleated cells and present endogenous antigen such as virus proteins, to the CD8+ cytotoxic T cells.

The antigen / HLA complex is recognized by TCR on the T cell (Figure 3 on page 27). This receptor is a heterodimeric molecule and comprises α and β chains in the majority of T cells. The α and β gene regions, encoding the respective components of the TCR, include a large pool of categorized gene segments. During ontogeneity, T cells rearrange these segments to express
functional TCR, which together with highly random imprecision in the joining of segments potentiate an enormous number of different TCRs (Roitt et al. 1996). This ensures that T cells in an individual have the capacity to respond against different pathogens encountered during lifetime.

In addition to HLA, TCR and antigen, the contact between APC and T cell also includes a number of other molecules (Figure 3 on page 28). These accessory molecules deliver costimulatory signals and enhance adhesion between the cells, promoting T cell activation (Wingren et al. 1995). Costimulatory signals are especially needed in the activation of naive T cells (Croft et al. 1994, Dubey et al. 1995, Dubey et al. 1996). CD28 on T cells, recognizing B7 on APC, delivers a positive signal for T cell activation together with the TCR signal (June et al. 1994). In addition, the enhanced expression of ICAM-1 on APC, interacting with LFA-1 on the T cell, stimulates adhesion and T cell proliferation (Wingren et al. 1995). T cell activation induces the expression of CTLA-4, which interact with B7 and conduct an inhibitory signal for the termination of T cell activation (Bugeon and Dallman 2000).

**Role of cytokines.** The adaptive immune system initiated by activated Th cells is modulated by cytokines, which allow proliferation and differentiation of precursor cells into specific effector cells. T cells can polarize towards Th1 and Th2 type cells, which are characterized by the cytokine profiles they produce. Th1 cells produce IL-2, INFγ and TNFα/β. These proinflammatory cytokines promote T cell-mediated cytotoxicity and phagocytosis by macrophages. Th2 type cytokines such as IL-4, IL-5 and IL-10 promote B cell differentiation and antibody production. Th1 and Th2 subpopulations are mutually inhibitory via the cytokines they produce (Roitt et al. 1996). In addition, Th2 type cytokines are anti-inflammatory and are involved in the termination of an inflammatory response (Roitt et al. 1996).

### 2.2.1 Breakdown of T and B cell tolerance

Pathogenic T cells in MS may recognize structures which are typical to the CNS, for example myelin basic protein (MBP), proteolipid protein and myelin oligodendrocyte protein (MOG) (Martino and Hartung 1999). Myelin-specific T cells are seen both in patients with MS and healthy individuals, although these T cells are more activated or differentiated in the memory T cells in patients (Martino and Hartung 1999). This suggests that the immunological tolerance of the cells has been broken down in subjects with MS. Further, breakdown of B cell tolerance may occur, since autoantibodies against myelin components have been detected in MS lesions (Genain et al. 1999). As B-cell activation by protein antigen is usually T-cell-dependent, synergistic interaction of T and B cell responses may operate in MS (Archeilos et al. 2000). It is generally assumed that
autoreactive T and B cells may be activated in peripheral lymphoid tissue and pass into the circulation as dormant memory cell clones, which can subsequently be reactivated and migrate into the CNS.

**Mechanisms of T and B cell tolerance breakdown.** In the periphery autoreactive T cells may be activated during microbe infection, if microbial and CNS antigen share antigenic determinants (molecular mimicry). This conception is supported by the finding that myelin-specific T cell clones are activated by several viral peptides in vitro (Wucherpfennig and Strominger 1995). In addition, for some MBP peptide-specific T cells antigen recognition is highly degenerate and many peptide ligands from self and microbial antigens are more potent stimulants than MBP (Hemmer et al. 1997). On the other hand, activation of autoreactive T cells as well as autoreactive B cells may be a random by-stander effect of the cytokines produced by an intensive inflammatory response (Tough et al. 1996). Non-specific T cell activation and autoimmunity may also occur by microbial superantigens (Schiffenbauer et al. 1998), which can form a functional bridge between T cells and APC. Activation of autoreactive B cells may also take place by molecular mimicry and superantigens (Archelos et al. 2000), as in the case of T cells. In addition, immunological tolerance may be broken down during an attack against myelin in the CNS by the epitope spreading, where polyclonal immune responses are developed against additional cryptic determinants of myelin (Lehmann et al. 1992). On the other hand, CNS may have such sequestered self-antigens which have never toleraised in thymic negative selection. An immune response against a pathogen in the brain may release neural antigens into the local lymphoid tissue which prime autoaggressive immune cell clones in the periphery and can target the antigen source in the brain (Hemmer et al. 2002).

**2.2.2. Immune responses against myelin and oligodendrocytes**

**Immune responses against myelin.** Active inflammatory lesions of MS include mainly T cells, some B cells and plasma cells, activated macrophages and microglia cells (Lassmann et al. 1998). Both Th1 type cytokines, such as IL-2, TNFα/β and INFγ, and Th2 type cytokines such as IL-4 and IL-10, have been demonstrated in MS lesions (Cannella and Raine 1995). Activated autoreactive CD4+ T cells and activated microglia cells may produce proinflammatory cytokines, which enhance different inflammatory mechanisms in the CNS (Figure 1 on page 15). Activated macrophages may phagocyte myelin, produce toxic substances and act as APCs in the lesions (Noseworthy et al. 2000). Specific cytotoxic T cell responses to different myelin components may play a role in demyelination (Tsuchida et al. 1994). A study using single-cell polymerase chain reaction showed that two MS patients had a clear clonal expansion of CD8+ T cells but not CD4+ T cells (Babbe et
al. 2000), suggesting that one major antigen drives immune responses by CD8+ cells in the CNS. Likewise, clonal expansion of B cells in the lesion and CSF in the CNS of patients with MS suggest a focused autoantibody response in demyelination (Baranzini et al. 1999, Colombo et al. 2000, Qin et al. 1998). For example, MOG- and MBP-specific B cell responses may be involved in the immunopathogenesis of the disease in a subset of MS patients (Reindl et al. 1999). Autoantibodies may have several effector mechanisms, such as complement activation and myelin damage in the MS lesion (Piddlesden et al. 1993, Storch et al. 1998) and receptor-mediated phagocytosis of myelin by macrophages (Lucchinetti et al. 1998).

**Immune responses against oligodendrocytes and axons.** In addition to myelin, myelin-producing cells, oligodendrocytes and their progenitor cells, are a target and prone to die in MS lesions (Figure 1). The reduced availability of oligodendrocytes in the MS lesion results in impaired capacity of remyelination. Cytotoxic T cells producing perforin may cause lysis of oligodendrocytes (Scolding et al. 1990, Zeine et al. 1998). TNF is cytotoxic for oligodendrocytes and may directly cause the death of these cells, since the expression of TNFα is correlated with oligodendrocyte pathology and demyelination activity (Bitsch et al. 2000a, Probert et al. 2000). Reactive oxygen and nitrogen species produced by macrophages and microglia during an inflammation may also cause oligodenroglial cell death in the MS lesion (Smith et al. 1999). In addition, oligodendrocytes may undergo apoptotic cell death bysignalling via their Fas-receptor (D'Souza et al. 1996, Sabelko-Downes et al. 1999). In the case of axonal damage and neurodegeneration, studies have only recently commenced, one finding suggests that macrophages and cytotoxic T cells may attack axonal antigens (Bitsch et al. 2000b).

**A regulatory defect of the immune system** may explain the sustained inflammation which occurs in the CNS of patients with MS (Hemmer et al. 2002). A defect in the production of Th2-associated cytokines may be a factor in the initiation and propagation of CNS inflammation in the disease, since Th2-type cytokines have been connected with suppression or recovery in MS and in a mouse model of experimental allergic encephalomyelitis (EAE) (Cua et al. 1999, Cua et al. 2001, Kennedy et al. 1992). In addition, reduced activity of the suppressive T cells may be implicated in the control of activation of autoreactive T cells (Hartung 1995). It has also been suggested that genetically determined failure of activation-induced apoptosis of autoreactive T cells in the CNS may be a cause of MS (Pender 1998). In general, genetic factors affect the quantity and quality of the immune response and may be important in the formation of unbalanced immune responses.
Figure 1. General features of a multiple sclerosis lesion. Autoreactive T and B cells are activated in the periphery, for example during an infection. Activated lymphocytes penetrate across the blood-brain barrier (BBB) and migrate into the central nervous system (CNS). Activated antigen-presenting cells (APC) can present antigen to the Th cells, which can lead to reactivation of the Th cells. Specialized Th1 and Th2 cells can arise. Th1 cells secrete cytokines which help macrophages to destroy the myelin and activate effector functions of cytotoxic T cells (Tc). Th2 cell-produced cytokines promote B cell differentiation and activate antibody production by plasma cells (PC). Antibodies may facilitate myelin degradation together with complement as well as myelin phagocytosis by macrophages. Immune responses by macrophages, Tc cells and TNFα may also attack oligodendrocytes. Naked axons may also be vulnerable to inflammatory processes in the lesion.
2.3 Immunopathogenesis of MS

Anatomically the blood-brain barrier (BBB) limits the entrance of macromolecules and cells from the peripheral circulation into the brain, which is one important reason for the relatively low immunological activity in the brain. Further, electrically active neurons strongly down-regulate MHC expression on their own membrane and surrounding glial cells (Neumann et al. 1995), which favors reduced immune responses to stimulus. However, activated T cells may initiate the brain inflammation in MS, since they have the capacity to cross the intact blood-brain barrier (BBB) (Hickey 1991). On the other hand, loss of CNS tissue integrity may activate innate immunity of the CNS, in which microglial cells produce cytokines and may thus initiate or exacerbate the inflammatory response in MS (Hemmer et al. 2002)

Transmigration of immune cells. Fresh T cells isolated from MS patients evince enhanced expression of several adhesion and activation molecules (Elovaara et al. 1998, Lou et al. 1997, Svenningsson et al. 1993, Teleshova et al. 2000), this possibly being linked to the capacity of T cells to migrate into the brain tissue. The intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) together with their ligands, the leukocyte function-associated antigen-1 (LFA-1) and the very late antigen-1 (VLA-4), facilitate endothelial adhesion of leukocytes and their expression is increased in MS lesions (Cannella and Raine 1995, Lee and Benveniste 1999, Sobel et al. 1990, Washington et al. 1994). Secreted chemoattractant molecules drive the extravasation. Chemokine pathways of RANTES and chemokine receptor-5 (CCR5) as well as IP-10 and CXC chemokine receptor-3 (CXCR3), associated with T helper 1 (Th 1) type responses (Qin et al. 1998), have been detected in MS lesions (Balashov et al. 1999, Baranzini et al. 2000, Boven et al. 2000, Sorensen et al. 1999) and blood cells of MS patients (Jalonen et al. 2002). In addition, expression of plasminogen activator and matrix metalloproteases and their endogenous inhibitors has been demonstrated in the evolution of MS lesion (Cuzner et al. 1996, Gveric et al. 2001, Lindberg et al. 2001). This would suggest that proteolytic activity is needed to degrade the basement membrane and extracellular matrix to open the way for the lymphocytes to enter the tissue.

Lymphocyte reactivation in brain tissue. Infiltrated T and B cells need to be reactivated in the CNS to persist, since non-activated immune cells may undergo apoptotic cell death there (Pender and Rist 2001) or return to the circulation. Antigen-dependent activation of T helper cells by an APC together with cytokine signals may be crucial in the initiation or sustance of brain inflammation in MS. This conception is supported by the finding that CD4 and HLA DRα transcripts are dramatically increased in brain samples from MS patients as compared to controls
Perivascular macrophages may be the first cells supporting T cell activation (Lassmann et al. 1991). Microglia cells and astrocytes may also have a capacity to present antigen, since cytokines produced by ongoing-inflammatory in the CNS can up-regulate HLA and accessory molecule expression in these residental cells (Antel and Owens 1999). An immune response in the brain may lead to enhanced production of primary inflammatory cytokines and activation and damage of the BBB, which can allow massive secondary cell infiltration and leakage of macromolecules such as autoantibodies and complement into the brain.

**Benefical role of inflammatory mediators in the lesion.** Inflammatory cytokines in an MS lesion may induce protease expression by immune cells such as macrophages (Opdenakker and Van Damme 1994). Proteases may be harmful, since proteolytic activity is linked to myelin destruction in MS in vitro (Proost et al. 1993). On the other hand, proteases may be needed in axonal outgrowth (Campbell and Pagenstecher 1999), which may allow restoration of the function of injured axons in MS. Overall, cytokines and neurotropic factors produced by infiltrating immune cells and glial cells in the demyelinating lesion may be not only harmful but also useful, since they may provide protection and contribute to tissue repair (Antel and Owens 1999).

### 3. Genetics of MS

#### 3.1. Genetic versus environmental factors in susceptibility

MS is a common condition, its prevalence exceeding 30 cases in 100,000 population (>30 / 10^5), in northern Europe, the northern United States, Canada, southern Australia and New Zealand (Compston 1999). The prevalence is low (<5 / 10^5) in uncharted regions, Asia and South America. Medium prevalence regions (5-30 / 10^5) are southern Europe, the southern United States and northern Australia. Geographical distributions of MS can be explained either by population genetics or environmental factors (Compston 1999). Migration studies have shown that some environmental factors before the age of 15 have a role in disease susceptibility (Kurtzke et al. 1985). Viruses may play a role, since MS relapses are often associated with common virus infections (Panitch 1994). Moreover, increased titers of viral antibodies, particularly to measles virus, have been found in the serum and CSF of MS patients (Cook et al. 1995). By genomic methods, there is evidence that human herpesvirus-6 (Soldan et al. 1997), Epstein-Barr virus (Wandinger et al. 2000) and Chlamydia pneumoniae (Wandinger et al. 2000) may be linked with MS.

MS is usually a sporadic disease, although familial clustering is commonly recognized. In Finland, an exceptionally high familial occurrence of MS has been reported in the Seinäjoki district from
1965 to 1993, suggesting that the frequency of susceptibility genes is high in that section of the population (Sumelahti et al. 2001). Family and twin studies have shown that the concordance rate of MS for monozygotic twins is about 30% as against about 2–5% for dizygotic twins and siblings (Ebers et al. 1986, Mumford et al. 1994, Sadovnick et al. 1993). In a Finnish twin cohort of 15,815 pairs, the concordance rate of MS was 29% in monozygotic twins and the discordance rate 100% in dizygotic twins (Kinnunen et al. 1988). These studies indicate that both genes and environmental factors are involved in the disease etiology.

Taken together, MS appears to be a complex trait in which several genes and environmental factors together with stochastic events, somatic mutations and other components of change (Ebers 1994) are involved in the development of the disease. Many environmental factors have been considered, for example general levels of infection, climate, changing socio-cultural factors, dietary effects and emotional stress (Ebers 1998). It may be that a certain number of risk factors, both genetic and environmental, might set the threshold for the development of clinical MS.

3.2. Strategies in searching for susceptibility genes

Genetic polymorphisms occur throughout the human genome on the average once every 500-1000 base pairs. The different DNA sequences of genes are referred as alleles. Inherited alleles of a gene or genes form the genotype of an individual. Genetic polymorphisms can be of several types, the most common being a single nucleotide polymorphism or SNP where one base is replaced by another. Insertions of additional sequences or deletions can also occur. Microsatellite polymorphisms have repetitive sequences of variable length. Especially polymorphisms on the regulatory and coding regions of a gene can have an effect on the expression or function of the protein. Overall, certain alleles or genotypes may constitute risk factors in the development of a disease.

Candidate gene approaches seek susceptibility genes contributing to disease expression. Selection of them is based on the hypothesized pathogenetic model of the disease. Candidate genes are tested in association or in linkage analyses. A population-based association study compares allele or genotype frequencies between unrelated MS patients and ethnically matched normal subjects. The statistical power of this method is relatively good, but it is vulnerable to ethnicity and regional genetic differences in a population. Family-based association analyses circumvent these problems; the parental alleles transmitted to patients are compared to those not transmitted to the patients. Linkage analyses follow gene segregation in families. Because linkage focuses only on recent ancestry, where there have been relatively few opportunities for recombination to occur, the
identified disease gene regions may be extensive (Cardon and Bell 2001). However, this method is not statistically so powerful as the case-control analyses. Whole-genome screens allow gene search without an *a priori* hypothesis of a disease, since it uses evenly distributed genetic markers in the genome. Usually 350-450 polymorphic markers are screened in a genome screen, which is tested in families.

3.3. Candidate gene studies of MS

As one crucial step in the pathogenesis of MS seems to be immune responses against myelin, genetic association and linkage studies in MS have mainly focused on HLA and non-HLA genes of the immune system and the myelin basic protein gene. Table 2 (on page 25) gives a concise overview of candidate gene studies in MS. A suggested function of the selected genes in MS is presented in Figure 3 (on page 27).

3.3.1. Human leukocyte antigen genes

**Genes encoding HLA alleles.** HLA class I and II molecules are encoded by the HLA region on chromosome 6p21.3 (Figure 2 on page 20). HLA class I molecules consist of an α-chain and β2-microglobulin (encoded on chromosome 15). α-Chains are encoded by three loci, HLA-A, HLA-B and HLA-C, all of which have several alleles. HLA class II molecules consist of α and β chains, which are encoded by HLA-DP, HLA-DQ and HLA-DR loci. The HLA-DR locus contains three functional genes: one α and several β genes, whereas the HLA-DQ and HLA-DP loci each have one α and one β gene. β genes of these three loci possess many alleles and α genes some. Polymorphic sequences of HLA genes are mostly situated in the amino-terminal region, which encodes the antigen-binding groove of the molecule (Lechler and Warrens 2000). The response depends on the ability of specific pockets in the groove to bind certain peptides and no others. This is the basis for antigen-specific control of the immune response. Between HLA class I and II genes are located HLA class III genes, which involve gene loci for complement, heat shock proteins and TNFα/β (Figure 2). Overall, the HLA region is the most polymorphic and gene-dense in human genome, having over 200 identified loci (The MHC sequencing consortium 1999). It is estimated that about 40% of the MHC region genes encode the components of the immune system (The HLA sequencing consortium 1999). In addition, characteristic for this region is an especially strong linkage disequilibrium between the genes, which is the basis of specific HLA haplotypes.
**Figure 2.** Simplified diagram of the human leukocyte antigen complex on chromosome 6.

**HLA DR2 and MS.** Association studies have well demonstrated in Caucasians that MS is associated with the HLA DR2 haplotype (Rasmussen et al. 2001a). In addition, two out of four whole genome screens in MS families (Ebers et al. 1996, Haines et al. 1996, Kuokkanen et al. 1997, Sawcer et al. 1996) confirmed the importance of the HLA region (Haines et al. 1996, Sawcer et al. 1996). The MS-associated HLA DR2 haplotype is now established to consist in DRB1*1501, DRB5*0101, DQA1*0102 and DQB1*0602 alleles (Fogdell et al. 1995). These alleles are in strong linkage disequilibrium and no primary susceptibility allele has been resolved. This haplotype increases the risk of MS about 2 – 4-fold in both sporadic and familial MS (Oturai et al. 1999). In addition, carriers of the DRB1*15-allele have disease onset at an earlier age than non-carriers, suggesting that this allele increases the genetic loading of an individual for MS (Masterman et al. 2000; Weatherby et al. 2001). Moreover, a meta-analyses from three northern European studies suggest that the effect of the DRB1*15 haplotype is additive in MS (Rasmussen et al. 2001). It is of note that some studies suggest that certain HLA alleles such as DR1 (Francis et al. 1991, Ilonen et al. 1983, Kinnunen et al. 1984, Madigand et al. 1982, Runmarker et al. 1994), DR6 (Haegert et al. 1996, Laaksonen et al. 2002, Madigand et al. 1982) and DR7 (Haegert et al. 1996, Madigand et al. 1982, Spurkland et al. 1991) may be protective against MS independently of the DR2 association. However, the significance of findings in this respect is not clear.

The DRB1*1501 allele is not associated with Asian-type MS, which is also characterized by a different pathology than the Western type of MS (Kira et al. 1996). In addition, Sardinians, who have a relatively low frequency of the MS-associated DR2 haplotype in both patients and normal population, have the DR4 haplotype as a susceptibility factor in MS (Marrosu et al. 1988a, Marrosu et al. 1998b). These observations suggest that MS may be immunogenetically different in different populations.

It seems that the disease allele of the HLA DR2 haplotype is situated in the region limited by the DP or DQA2 loci in the centromeric side of the DR locus and by C4/CYP21 within the class III
region (Hillert and Olerup 1993). This maps the primary disease allele relatively close to the DR2 locus, or it may be one of the known alleles in the MS-defined DR2 haplotype. On the other hand, certain HLA class I alleles have been shown to modulate the risk conferred by DR2 (Fogdell-Hahn et al. 2000), suggesting that the synergy of specific alleles in the HLA region plays a role in MS susceptibility. In addition, two studies have found that the Val 86 / Val 86 genotype of HLADRB1-alleles (including 1501, 0301 and 1301) is associated with MS susceptibility independently of the DRB1*1501 effect (Allen et al. 1994, Teutsch et al. 1999). This would imply that specific amino acids in the antigen-binding groove may have a functional role in the binding and presentation of potential autoantigens such as peptides from MBP.

**Other HLA genes and MS.** The HLA class II region also contains genes encoding proteasome components (LMP-1 and LMP-2) and peptide transporter proteins (TAP-1 and TAP-2) involved in the HLA class I antigen-processing pathway. TAP and LMP genes have not been found to be significantly associated with MS susceptibility (Bell and Ramachandran 1995, Bennetts et al. 1995, Kellar-Wood et al. 1994, Liblau et al. 1993, Spurkland et al. 1994, Vandevyver et al. 1994). In addition, the Notch gene, which is situated in close proximity to the HLA class II region, is not associated with MS in simplex families (Broadley et al. 2001). Extended studies of the HLA region III have focused on a gene for TNF\(\alpha\), since it seems to be important in MS pathology, and TNF\(\alpha\) – 308 biallelic promoter polymorphism may have an effect on the levels of TNF production (Louis et al. 1998). However, there is only one positive report concerning this gene and MS susceptibility (Kirk et al. 1997) and a number of negative reports in both susceptibility and severity (Fugger et al. 1990, Garcia-Merino et al. 1996, He et al. 1995, Lucotte et al. 2000, Maurer et al. 1999, Oturai et al. 1999, Roth et al. 1994, Sandberg-Wollheim et al. 1995, Weisshenker et al. 1997, Wingerchuk et al. 1997). In contrast, one study suggests that TNF –376 promoter polymorphism is associated with MS susceptibility independently of the HLA DRB1*1501 association (Fernandez-Arquero et al. 1999). Taken together, currently it seems that the HLA-DR-DQ subregion determines primary susceptibility to MS in the HLA region.

HLA class II associations are characteristic for many autoimmune diseases, among them type 1 diabetes and rheumatoid arthritis. Also, MS association with the HLA class II region is an important pointer to an autoimmune etiology of MS. One simple conception is that the disease-associated HLA alleles can present such antigenic peptides which elicit an immune response against the target tissue and induce autoimmunity. In a transgenic mouse T-cells specific for myelin in the context of HLA DR2 have been demonstrated to be sufficient and necessary for an induction disease resembling MS (Madsen et al. 1999).
3.3.2. T-cell receptor genes

Genes for TCR α genes are situated on chromosome 14 and β genes on chromosome 7q35. Both chromosomal regions have polymorphic markers, which could affect the function of TCR and are potentially a resource of autoreactive T cells. Numbers of studies concerning TCR gene polymorphisms have been conducted in respect of susceptibility of MS. For TCRα, one positive finding has been reported (Oksenberg et al. 1989), but other studies have not been able to confirm the initial finding (Droogan et al. 1996, Eoli et al. 1994, Hashimoto et al. 1992, Hillert et al. 1992, Lynch et al. 1992, Vandevyver et al. 1994a). For TCRβ, reports have been more conflicting, showing both positive (Beall et al. 1993, Beall et al. 1989, Buhler et al. 2000, Charmley et al. 1991, Epplen et al. 1997, Hockertz et al. 1998, Martinez-Naves et al. 1993, Seboun et al. 1989) and negative results (Droogan et al. 1996, Fugger et al. 1990b, Hillert et al. 1991, Lynch et al. 1991, Vandevyver et al. 1994, Wansen et al. 1997, Wei et al. 1995). These discrepancies may in part imply that the TCRβ gene is a susceptibility factor in certain ethnically or genetically stratified MS populations. Indeed, some studies have suggested that HLA DR2 status could have an effect on the MS risk conferred by the TCRβ gene (Beall et al. 1993, Buhler et al. 2000, Epplen et al. 1997, Hockertz et al. 1998).

3.3.3. Genes of accessory molecules involved in antigen presentation

The ICAM-1 gene on chromosome 19 has been found to be associated with MS in a Polish population (Mycko, 1998 #909), but no association with this gene was found in Dutch (Killestein et al. 2000) and Sardinian populations (Marrosu et al. 2000). The CTLA-4 gene on chromosome 2q33 has exon 1 polymorphism, which reduces the inhibitory function of CTLA-4 (Kouki et al. 2000). Moreover, in this gene polymorphic sites on promoter and exon 1 influence gene expression (Ligers et al. 2001). Studies from Sweden and Norway have found an association between a functional polymorphism of exon 1 on the CTLA-4 gene and MS susceptibility (Harbo et al. 1999, Ligers et al. 1999), although a study analyzing European Caucasians was not able to confirm the association (Rasmussen et al. 2001b). CD45 is a transmembrane protein-tyrosine phosphatase, which in T cells maintains the TCR in a primed state, allowing TCR activation upon contact with APC. A functional point mutation of the gene encoding CD45 (Lynch and Weiss 2001) has been associated with MS susceptibility in three out of four independent case-control studies (Jacobsen et al. 2000). However, a British study does not support the finding (Vorechovsky et al. 2001). It is of interest that the T cell-specific adapter protein controlling T cell activation was associated with MS in a Norwegian population (Dai et al. 2001). Taken together, CTLA-4 and CD45 genes as potential susceptibility
Factors in MS support the conception that dysregulated T cell response may contribute to the development of MS.

3.3.4. Cytokine genes

Increased production of both Th1 and Th2 cytokines is seen in CSF cells (Calabresi et al. 1998, Monteyne et al. 1998) and lesions (Baranzini et al. 2000, Cannella and Raine 1995) from MS patients as compared to CSF cells from healthy controls or normal CNS tissue, respectively. Thus, cytokine gene polymorphisms may have an effect on the susceptibility, course or severity of MS.

Cytokine genes and MS. IL-1 region genes have been held to be associated with the susceptibility to or severity of MS (de la Concha et al. 1997, Schrijver et al. 1999, Sciacca et al. 1999, Semana et al. 1997), although numbers of negative reports have also been published (Epplen et al. 1997, Huang et al. 1996, Niino et al. 2001, Wansen et al. 1997). A study concerning IL-4 and MS found that an intronic polymorphism of IL-4 modifies the age of MS onset (Vandenbroeck et al. 1997) and a moderate association has been found between a microsatellite marker of IL-4 receptor and PPMS (Hackstein et al. 2001). The IL-6 gene was not associated with MS susceptibility (Fedetz et al. 2001), but this gene may influence the disease course (Vandenbroeck et al. 2000). In a Sardinian study the INF-γ gene was found to be associated with MS in DR3/DR4-negatives (Vandenbroeck et al. 1998), but no association was found with this gene and in Nordic multiple sclerosis patients (Dai et al. 2001). Also, INF-α is a potent susceptibility gene in MS (Miterski et al. 1999). The TGF-β1 gene may affect disease expression (Green et al. 2001), although it is not associated with disease susceptibility (Weinshenker et al. 2001). TGF-β2, IL-4R, INF-γ (He et al. 1998) and IL-2 (Matesanz et al. 2001) may be of significance in MS, although another study analyzing fifteen Th1 and Th2 cytokine genes found no linkage with MS (Reboul et al. 2000).

As noted above, findings concerning cytokine genes and MS are conflicting, which would suggest that the role of the different cytokines may vary in different genetic backgrounds of individuals and in different subtypes of the disease. Further, the immunology of MS may be complex, as suggested by studies in which no clear shift of cytokine profile was seen in pathology (Baranzini et al. 2000, Monteyne et al. 1997) or after intervention (Duddy et al. 1999, Khademi et al. 2000, Wandinger et al. 2001). The network effects of cytokines with each other and with other inflammatory factors may underlie the complexity, which thus presents a problem in searching for causal relationships between cytokines and diseases (Callard et al. 1999). However, a repeated finding of functional cytokine polymorphisms in MS even in one population could give a valuable clue to the pathogenesis of this disease.
3.3.5 Autoantigen genes

Structural protein components of myelin may serve as an autoantigen in MS. For example, genes encoding these components may underline unbalanced protein production or a structural deficit of myelin, which could trigger an immune response against myelin in MS. MBP is a major structural protein component of CNS myelin and a popular autoantigen candidate in MS. The MBP gene at chromosomal location 18q22-q23 has a repetitive sequence 5' to the MBP gene. A Canadian study has found an association between this region and MS susceptibility (Boylan et al. 1990). In a Finnish study both association and linkage to the region have likewise proved significance (Tienari et al. 1998, Tienari et al. 1992), as also suggested by Danish (Ibsen and Clausen 1996) and Italian (Guerini et al. 2000) studies. A Swedish study again, found no association between this region and MS (He et al. 1998). Moreover, numbers of other studies focusing on other regions of the MBP gene have reported negative findings (Barcellos et al. 1997, Seboun et al. 1999, Wood et al. 1994). Nonetheless, the 5' flanking region of the MBP gene or another gene in its vicinity may be a susceptibility factor for MS in some populations. Attention has also focused on the MOG gene, which is telomeric to the HLA region. MOG is a minor protein of myelin, but is expressed on the outer surface of myelin and may serve as an autoantigen in MS. However, no association of MOG with MS has been found (Rodriguez et al. 1997, Roth et al. 1995). Similarly, the myelin-associated glycoprotein gene endoding another minor protein has not been associated with MS (Chataway et al. 1999).
Table 2. A concise overview of the studied immunological candidate genes in susceptibility to MS (table continues over)

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<td>(Guerini et al. 2000)</td>
</tr>
<tr>
<td>MAG</td>
<td>19q13.1</td>
<td>British</td>
<td>(Chataway et al. 1999)</td>
<td></td>
</tr>
</tbody>
</table>

Abbrevations: TCR, T-cell receptor; CTLA-4, cytotoxic T lymphocyte antigen-4; ICAM-1, intercellular adhesion molecule-1; IL-1RA, interleukin-1 receptor antagonist; CCR5, chemokine receptor-5; MCP-3, monocyte chemotactic protein; TIMP-3, tissue inhibitor of metalloproteinase-3; MBP, myelin basic protein and MAG, myelin associated glycoprotein.
Figure 3. A model of molecules involved in the activation of autoaggressive CD4+ T cells.

Genes encoding the molecules involved in the contact between T cell and antigen-presenting cells may in part underline the development of myelin basic protein-specific T-cells in susceptibility to MS. In this hypothesized model the T-cell receptor (TCR) complex recognizes a peptide derived from myelin basic protein (MBP) lodged in the groove of the HLA DR2 molecule. Upon TCR stimulation CD4-associated CD45 phosphatase activity is a very early biochemical event triggered. In addition, B7 reinforces the signal from TCR and induces positive activation of T cells. Cytokine signals also regulate the T cell response. Interactions of intercellular adhesion molecule-1 (ICAM-1) and lymphocyte function antigen-1 (LFA-1) and vascular cell adhesion molecule (VCAM) and very late antigen-4 (VLA-4) mediate adhesion between the cells. Cytotoxic T lymphocyte antigen-4 (CTLA-4) expression is increased in activated T cells and signals for the termination of the response. Genes shown in bold have been associated with susceptibility to MS in at least two independent studies. Overall, the DR2 allele and other gene polymorphism(s) may develop such binding energetics, which can activate autoreactive T cells and autoimmunity in MS.

3.4. Whole-genome screens in MS

Several whole genome screens have now been performed in MS (Ebers et al. 1996, Haines et al. 1996, Kuokkanen et al. 1997, Sawcer et al. 1996, Sawcer et al. 2002). The screens found no strong chromosomal region in linkage with MS susceptibility. Several moderate links to MS were found, although they were heterogeneous between the screens. In the Canadian screen it emerged that regions of interest are 2p, 3p, 5p 11q and Xp (Ebers et al. 1996) and in the US-French screen they were 6p, 7q, 11p, 12 q and 19q (Haines et al. 1996). The UK screen found significant linkage with
1cen, 5cen, 6p, 7p, 14q, 17q and Xp (Sawcer et al. 1996). In addition, another UK genome screen using DNA pooling was able to confirm disease associations with regions 6p, 17q and 1p (Sawcer et al. 2002). In the relatively small Finnish screen found no statistically significant regions but positive linkage with regions 6p21 and 5p14-p12 (Kuokkanen et al. 1997). Clearly, further studies are needed for any other loci than 6p to confirm the importance in MS. One conclusion to be drawn from the screens is that many genes with minor additive and synergistic effects are obviously involved in MS susceptibility.

3.5. **Candidate genes associated with disease severity**

Genetic studies in MS have mainly focused on the search of susceptibility genes. However, during the recent years it has become clear that genetic component may also contribute to the progression and severity of the disease. Apolipoprotein (Apo) E is an important molecule in several biological processes. A primary metabolic role for apoE is to transport and deliver lipids from one tissue or cell type to another. Apo E has three alleles, apoE4, E3 and E2 at a single gene locus. ApoE4 allele is associated with the increased risk for atherosclerosis and Alzheimer's disease. In MS, the presence of the apoE4 allele has been held in several studies to be an unfavorable prognostic factor (Chapman et al. 2001, Evangelou et al. 1999, Fazekas et al. 2001, Fazekas et al. 2000, Hogh et al. 2000, Schmidt et al. 2002), although conflicting reports have also been published (Ferri et al. 2000, Pirttila et al. 2000, Weatherby et al. 2000). ApoE may participate in repair processes within the CNS and the E4 allele may be linked to reduced capacity for remyelination.

Two studies suggest that SNP of promoter region of IL-1β gene affects the prognosis of MS (Kantarci et al. 2000, Schrijver et al. 1999), although a study focusing on another SNP of the promoter region reported conflicting results (Feakes et al. 2000). In the IL-1 region, a tandem repeat polymorphism in intron 4 of the IL-1RA gene may also be of importance as a disease-modifying gene in MS, since the presence of allele 2 of this polymorphism in an individual is found to be a favorable prognostic factor (de la Concha et al. 1997, Feakes et al. 2000, Sciacca et al. 1999). Such important immunological genes as B7, CTLA-4, interferon-γ, IL-10 and IL-4 have shown no significant association with the severity of MS, as presented in the good review article by Kantarci (Kantarci et al. 2002).
3.6. Genetic heterogeneity

Regarding the candidate gene and whole-genome screens in MS, the HLA region is the only consistent susceptibility factor is found connected with MS in different Caucasian populations. This may be explained in part by the considerable genetic heterogeneity of the disease as affected by ethnicity and geographical stratification. In addition, the genetic background of MS may be dependent on DR2 status, since further studies with the UK screen have revealed that DR2 positives and DR2 negatives had different regions with positive linkage (Chataway et al. 1998). Other obvious confounding factors are clinical variables such as disease course, severity and age at onset. Furthermore, the immunopathogenetic heterogeneity observed in MS lesions (Lucchinetti et al. 2000) may reflect in part the genetic variability on the disease. Overall, MS seems to be a heterogeneous disease entity in which multiple pathogenetic mechanisms may underlie the myelin destruction. A benefit of genetic studies is that they will allow a better understanding of different molecular pathways in the pathogenesis of MS, which could help to design rational therapies for different clinical and pathological subtypes of the disease.
AIMS OF THE STUDY

It seems that a central feature in the initiation and progression of MS pathogenesis is activation of autoaggressive T cells in the CNS and periphery. Previous genetic studies have shown the strongest association of MS with HLA class II region. As HLA class II molecules are most important in the thymic selection of and later antigen presentation to the class II-restricted CD4+ T cells, they may be a key in autoaggressive T cell activation. Furthermore, the activation of these cells may be facilitated by the induction of cytokines and the up-regulation of particular accessory molecules. HLA and non-HLA polymorphisms may thus influence the specificity and the nature of CD4+ T cell responses and autoimmunity. In the present work, we studied the contribution of such gene polymorphisms in susceptibility to and severity of MS in the Finnish population. This kind of genetic studies, using a genetically relatively homogeneous population, may be valuable in the search for genetic factors underlying a genetically heterogenous disease such as MS.

The specific aims of the study were

1) whether HLADRB1/3/4/5, ICAM-1, CTLA-4 and estrogen receptor I (ESRI) genes, involved in the contact between T cell and antigen presenting cell, contribute to MS (Studies I, IV ,V and VI).
2) whether cytokine signals by TNFα, IL-1 and IL-10 genes underlye the disease process of MS (Studies II and V).
3) whether genetic determined chemotactic or proteolytic abilities in relation to CCR5 and PAI-1 genes, play role in the development of MS (Studies III and V).
4) to assess the clinical relevance of immunogenetic findings on sex, severity, disease course and age at onset in MS (Studies I, II, III and V).
SUBJECTS AND METHODS

1. Patients

The study population comprised 116 patients from Tampere University Hospital with clinically definitive MS according to the criteria of Poser (Poser et al. 1983). Neurological disability was measured using the Kurtzke Expanded Disability Status Scale (EDSS) (Kurtzke 1983). Based on the EDSS, patients were classified as suffering a mild / moderate (EDSS 0 - 5.5) or severe form of MS (EDSS 6 – 10). This classification was used, since patients with EDSS 6 or more require walking assistance (Kurtzke 1983) and it is generally used in studies similar to ours. The diagnosis of patients were established by certified neurologist. The EDSS scoring of the patients was assessed for the study and they were performed by neurologist. MRI and laboratory tests of CSF were done to all patients to aid to establish the diagnosis or to determine the disease activity. Table 1 gives the clinical characteristics of patients.

Table 1. Clinical characteristics of MS patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (F:M)</td>
<td>1.4 : 1</td>
</tr>
<tr>
<td>Females, age (years), n = 67, mean ± SD (range)</td>
<td>46 ± 10 (26 – 78)</td>
</tr>
<tr>
<td>Men, age (years), n = 49, mean ± SD (range)</td>
<td>46 ± 10 (24 – 67)</td>
</tr>
<tr>
<td>Age at onset (years), n = 115, mean ± SD</td>
<td>32 (14 - 50)</td>
</tr>
<tr>
<td>Females, n = 67</td>
<td>32 (14 - 49)</td>
</tr>
<tr>
<td>Men, n = 48</td>
<td>32 (20 - 50)</td>
</tr>
<tr>
<td>Disease duration (years), n = 115, median (range)</td>
<td>13 (1 – 38)</td>
</tr>
<tr>
<td>Females, n = 67</td>
<td>13 (1 - 38)</td>
</tr>
<tr>
<td>Men, n = 48</td>
<td>14 (1 - 37)</td>
</tr>
<tr>
<td>Disease course, number (females / male)</td>
<td></td>
</tr>
<tr>
<td>Relapsing-remitting</td>
<td>43 (27 / 16)</td>
</tr>
<tr>
<td>Secondary progressive</td>
<td>43 (26 / 17)</td>
</tr>
<tr>
<td>Primary progressive</td>
<td>30 (14 / 16)</td>
</tr>
<tr>
<td>EDSS score, n = 112, median (range)</td>
<td>4.0 (0 – 8.0)</td>
</tr>
<tr>
<td>Females, n = 65</td>
<td>4.0 (0 - 8.0)</td>
</tr>
<tr>
<td>Men, n = 47</td>
<td>4.0 (0 - 8.0)</td>
</tr>
<tr>
<td>EDSS ≤ / &gt; 5.5</td>
<td>76 / 40</td>
</tr>
<tr>
<td>Females, n = 65</td>
<td>43 / 22</td>
</tr>
<tr>
<td>Men, n = 47</td>
<td>33 / 14</td>
</tr>
</tbody>
</table>

Abbrevation: EDSS, the Kurtzke Expanded Disability Status Scale

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2. Control subjects

Control group 1 (Studies I – VI)
A cohort of controls comprising 109 healthy Finnish subjects of the same sex and age distribution (aged 23 – 78, mean 46 ±10) as the MS patients were recruited, including medical staff (n = 83) and random Tampere residents (n = 26).

Control group 2 (Studies II and VI)
The controls comprised 400 healthy adults (18 – 60 years) who had donated blood in the Finnish Red Cross Blood Transfusion Centre, Tampere. These subjects served as controls in cytokine gene studies.

3. DNA extraction

DNA was extracted from whole blood using the QIAamp blood kit (Qiagen Inc, CA, USA) for MS patients and the control group of 109 subjects. DNA extraction was done by the salting-out method (Miller et al. 1988) for the control group comprising 400 subjects.

4. Genotyping

The selected polymorphisms on the candidate genes (Table 2) were genotyped by methods and primers previously described. HLA DRB1/3/4/5 –alleles were typed with the DR low-resolution kit using PCR and sequence-specific primers (Dynal A. S, Oslo, Norway). IL-10 –1082 polymorphism was detected by direct sequencing (Helminen et al. 1999). Polymerase chain reaction (PCR) and restriction enzyme digestion were utilized in the analysis of ICAM-1 (Vora et al. 1994), CTLA-4 (Donner et al. 1997), ESRI (Yaich et al. 1992), PAI-1 (Margaglione et al. 1997), IL-1 α/β (Bioque et al. 1995, di Giovine et al. 1992, McDowell et al. 1995) and TNF α (Wilson et al. 1992) polymorphisms. The digestion products of the amplified gene segments were detected by agarose gel electrophoresis and etidium bromide staining. IL-1RA (Tarlow et al. 1993) and CCR5 (Huang et al. 1996) alleles were produced by primer-specific PCR, which were run on agarose gel. Primer sequences and PCR programs used are shown in Table 3.
5. Statistical analyses

Frequencies of alleles and genotypes were compared between patients and controls by \(X^2\) or Fisher’s exact test. Associations between severity and gene polymorphisms were tested by three common methods. Logistic regression analyses and multiple regression analyses (SPSS 9.0 for Windows, SPSS Inc., 1999) were corrected for gender, age at onset and disease duration, since these factors are associated with adverse clinical outcome (Weinshenker et al. 1991). The independent samples t-test (SPSS 9.0 for Windows) was used to compare means of age at onset in MS patients stratified by genotypes or allele carrier status. The results were corrected for multiple testing in study of polymorphic regions of the IL-1 region on chromosome 2q and the HLA region on chromosome 6p. However, all the polymorphisms studied were not included in statistical corrections, since we considered our study explorative the aim being to screen for potential candidates for further studies. The criterion for the level of significance was set at \(p < 0.05\).
Table 2. Studied candidate genes of the immune system

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosomal region</th>
<th>Gene polymorphism</th>
<th>Potential function in MS pathogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA DRB1/3/4/5</td>
<td>6p21</td>
<td>Sequence polymorphism in exon 2</td>
<td>Present autoantigen to the T helper cell</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>19p13.2</td>
<td>Amino acid change; Lys/Gly 469</td>
<td>Extravasation of immune cells, autoreactive T helper cell activation</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>2q33</td>
<td>Amino acid change; Thr/Ala 49</td>
<td>Dysregulated T helper cell response</td>
</tr>
<tr>
<td>ESRI</td>
<td>6q25.1</td>
<td>Nucleotide change on intron</td>
<td>autoreactive T helper cell activation</td>
</tr>
<tr>
<td>TNFα</td>
<td>6p21</td>
<td>Nucleotide change on promoter</td>
<td>Promote inflammation</td>
</tr>
<tr>
<td>IL-1α (-889)</td>
<td>2q13-21</td>
<td>Nucleotide change on promoter</td>
<td>Unbalanced ratio of agonist and antagonist</td>
</tr>
<tr>
<td>IL-1β (-511)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β (+3953)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1RA (VNTR)</td>
<td></td>
<td>Variable number of tandem repeats</td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>1q31-q32</td>
<td>Nucleotide change on promoter</td>
<td>Deficient suppression of inflammation</td>
</tr>
<tr>
<td>CCR5</td>
<td>3p21</td>
<td>32-basepair deletion</td>
<td>T cell migration into the brain</td>
</tr>
<tr>
<td>PAI-1</td>
<td>7q21.2-23</td>
<td>Nucleotide insertion / deletion (4G / 5G) on promoter</td>
<td>Migration of immune cells into the brain, autoantigen production</td>
</tr>
</tbody>
</table>

Abbreviations: HLA, human leukocyte antigen; ICAM-1, intercellular adhesion molecule-1; CTLA-4, cytotoxic-T-lymphocyte antigen-4; ESR1, estrogen receptor 1; CCR5, chemokine receptor-5; IL-1, interleukin-1; IL-1RA, interleukin-1 receptor antagonist, TNFα, tumor necrosis factor-α, PAI-1, plasminogen activator inhibitor-1.
Table 3. Primers and PCR programs used in Studies I - VI (table continues over)

<table>
<thead>
<tr>
<th>Gene</th>
<th>PCR primers</th>
<th>PCR program</th>
<th>Cycles</th>
<th>RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA</td>
<td>Allele or group-specific primers (Dynal has not published the primer sequences)</td>
<td>94 C, (2 min), 45 s&lt;br&gt;64 C, 1 min 15 s&lt;br&gt;94 C, 45 s&lt;br&gt;61 C 1 min&lt;br&gt;72 C 45 s</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>5'-CCATCGGGGAATCAGTG-3'&lt;br&gt;5'-ACAGAGCACATTCACGGTC-3'</td>
<td>94 C, (4 min 30 s), 30 s&lt;br&gt;56 C, 30 s&lt;br&gt;72 C, 30 s, (5 min)</td>
<td>35</td>
<td>BstU1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTLA-4</td>
<td>5'-GCTCTACTTCTCCTGAAAGACCT-3'&lt;br&gt;5'-AACCCAGGTAGGAGAAACAC-3'</td>
<td>94 C, (4 min), 30 s&lt;br&gt;50 C, 30 s&lt;br&gt;72 C, 60 s, (4 min)</td>
<td>35</td>
<td>BbvI</td>
</tr>
<tr>
<td>ESRI</td>
<td>5'-CTGCCACCCTATCTGTATCT-3'&lt;br&gt;5'-TCTTTTCTCTGCCACCCTGGC-3'</td>
<td>94 C, (4 min), 1 min&lt;br&gt;57 C, 1 min&lt;br&gt;72 C, 1 min, (10 min)</td>
<td>30</td>
<td>PvuII</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>5'-AGGCAATAGGTTTTGAGG GCCAT-3'&lt;br&gt;5'-TCCTCCCTGCTCCGATTCCG-3'</td>
<td>(94 C, 3 min)&lt;br&gt;(60 C, 1 min)&lt;br&gt;(72 C, 1 min)&lt;br&gt;94 C, 1 min&lt;br&gt;69 C, 1 min&lt;br&gt;72 C, 1 min&lt;br&gt;(94 C, 1 min)&lt;br&gt;(60 C, 1 min)&lt;br&gt;(72 C, 5 min)</td>
<td>34</td>
<td>NcoI</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1α</td>
<td>5'-AAGCTTGTCTTACCACCTGAAC TAGGC-3'&lt;br&gt;5'-TTACATATGAGCCTTCCATG-3'</td>
<td>(96 C, 1 min),&lt;br&gt;94 C, 1 min&lt;br&gt;52 C, 1 min&lt;br&gt;72 C, 1 min, (4 min)&lt;br&gt;(55 C, 5 min)</td>
<td>40</td>
<td>NcoI</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5'-TGGCATTGATCTGGTAC-3'&lt;br&gt;5'-GT TTAGGAATCTTCCCACT-3'</td>
<td>(95 C, 2 min)&lt;br&gt;(55 C, 1 min)&lt;br&gt;(74 C, 1 min)&lt;br&gt;95 C, 1 min&lt;br&gt;55 C, 1 min&lt;br&gt;74 C, 1 min&lt;br&gt;95 C, 1 min&lt;br&gt;55 C, 1 min&lt;br&gt;74 C, 1 min, (4 min)</td>
<td>35</td>
<td>AvaI</td>
</tr>
<tr>
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</tr>
</tbody>
</table>
IL-1β 5'-GTTGTCATCAGACTTTGACC-3' 97 C, 2 min 3 Taq1
(+3953) 5'-TTCAGTTCATATGGACCAGA-3' 55 C, 2 min
97 C, 1 min
74 C, 1 min
97 C, 1 min 32
55 C, 1 min
74 C, 1 min
(73 C, 10 min)

IL-1RA 5'-CTCAGCAACACTCCTAT-3' (96 C, 1min 30 s) -
(VNTR) 5'-TCCTGGTCTGCAGGT-3' 94 C, 1 min 35
60 C, 1 min
70 C, 1 min
(72 C, 5 min)

IL-10 (-819) 5'-TAAATATCCTCAAAGTTCC-3' (95 C, 5 min) -
5'-ATCCAAGACAACACTACTAA-3' (56 C, 1 min)
(72 C, 1 min)
95 C, 1 min 29
56 C, 1 min
72 C, 1 min, (3 min)

CCR5 5'-CCTGGGCTGTCCATGCTG-3' 94 C, (4 min 30 s), 30 s 32 -
5'-GGCAGGACCAGCCCCAAGATG-3' 66 C, 30 s
72 C, 30 s, (10 min)

PAI-1 5'-CACAGAGAGAGTCTGGCCACGT-3' 95 C, (4 min 30 s), 30 s 32 Bsl I
5'-CCAACAGAGACTCTTTGGTCT-3' 60 C, 1 min
72 C, 30 s, (2 min)

Pre-denaturation and extra extension conditions are shown in parenthesis.
Abbreviations: HLA, human leukocyte antigen; ICAM-1, intercellular adhesion molecule-1; CTLA-4, cytotoxic-T-lymphocyte antigen-4; ESR1, estrogen receptor 1; CCR5, chemokine receptor-5; IL-1, interleukin-1; IL-1RA, interleukin-1 receptor antagonist; TNFα, tumor necrosis factor-α; PAI-1, plasminogen activator inhibitor-1 and RE, restriction enzyme.

RESULTS AND DISCUSSION

The well-known genetic association between HLA DR2 and MS suggests that Th cell activation by antigen is a crucial event in the immunopathogenesis of MS. Activated T cells initiate antigen-specific immune responses, which in the pathogenesis of MS may involve both cell-mediated and humoral responses. As HLA alleles underlie the specificity of antigen recognition, other molecules, especially those acting in the immunological synapse, may significantly modulate the level of T cell activation. Again, the immunogenetics of an individual may be a factor determining the result of the T cell response; whether it leads to Th1 or Th2 type responses, apoptotic cell death of the T cell or
anergic T cells. This may also mean that certain immunogenetic backgrounds may also increase the risk of chronic inflammatory or autoimmune diseases like MS. In the present study several immunologically relevant genes were tested in MS susceptibility and severity. Significant or tentative associations emerged in relation to genes of the HLA DRB1/3/4/5 region, ICAM-1, ESRI, PAI-1, IL-1RA and IL-10.

1. HLADRB1/3/4/5 alleles in susceptibility to MS

**DRB1*1501 allele.** We found a relatively strong association between the HLA DRB1*1501 allele and MS, at an odds ratio of 4.00. This finding was as expected in white individuals of northern European descent. In addition, the HLA DRB1*1501 allele tended to be a stronger risk factor for women than men, which is also suggested by other studies (Duquette et al. 1992, Hensiek et al. 2002). The biological mechanism underlying the association of the HLA DRB1*1501 allele with MS is not yet fully understood. Many possibilities have been presented (Oksenberg 1996). A common conception is that this allele can present myelin peptides to the autoaggressive CD4+ T cells and trigger autoimmunity against myelin in the CNS. On the other hand, this allele can bind with high affinity such viral or bacterial antigen peptides which have sequence homology with neural antigens. It is also possible that this allele has no crucial role in the genetic predisposition to MS, but is in linkage disequilibrium with a gene or genes which influence susceptibility.

**Other DRB1-alleles.** The frequency of the DR1 allele was significantly decreased in MS patients as compared to controls (OR=0.30, p(corrected) = 0.005). Three previous Finnish studies (Ilonen et al. 1983, Kinnunen et al. 1983, Laaksonen et al. 2002) as well as a number of other previous studies (Francis et al. 1991, Madigand et al. 1982, Runmarker et al. 1994) have also reported a protective association between the DR1 allele and MS. However, no significant association of this allele with MS in Finnish nuclear families was found in the analyses of relative predispositional effects of HLA alleles (Laaksonen et al. 2002). We also made an analysis of relative predispositional effects of HLA-DR alleles, and after excluding the DR2 allele, the DR1 allele still showed a significant association with MS (data not shown). In addition, in these analyses DR10, DR5 and DR3 were positively associated with susceptibility to MS. Our results suggest that other alleles than DRB1*1501 also have a role in MS susceptibility, although the effect of these is relatively small. In addition, the DR1 allele may have a small protective effect against MS.

**DR1 and DR53 alleles.** We found that the DR1 allele with the DR53 allele is highly protective against MS in all subjects (OR=0.09, p < 0.0001) and in DRB1*15-negative subjects (OR=0.09, p
(corrected) = 0.026). This suggests that synergism between HLA alleles rather than individual effects of the alleles play a role in MS protection. It is of note that previous studies have not taken into account the sum frequency of the DR1 and DR53 alleles, which need to be in focus in further studies to evaluate the potential interaction of DR53 and DR1 in MS protection. We found that in one large Swedish data-sets of MS patients and controls (Masterman et al. 2000), the sum frequency of DR4, DR7 and DR9, reflecting the frequency of DR53, was significantly decreased in MS patients as compared to controls (p<0.0001). However, frequency analyses of the DR53 allele in the carriers and non-carriers of the DRB1*15 allele could not be made from the published data. DR53 is a new and interesting candidate in the etiology of MS, since it evinces sequence homology with some EBV proteins as well as with many other common virus proteins (Dorak and Burnett 1994), although it is not known whether this mimicry is biologically functional. However, it is of interest that DR53 or the most common allele of the DR53 haplotype, namely DR4, is also associated with rheumatoid arthritis and leukemia (Dorak et al. 1995), in which EBV infection may equally play a role.

2. **No significant association of the non-HLA genes with MS susceptibility**

In the present study, MS susceptibility was not significantly associated with the polymorphisms on ICAM-1, CTLA-4, CCR5, ESRI, TNFα, IL-1α/β, IL-1RA, IL-10 and PAI-1 genes. However, in subgroup analyses we found interesting genetic differences between women and men. Furthermore, IL-10 gene polymorphism had an effect on the severity of MS.

3. **Sex differences in genetic susceptibility to MS**

The prevalences of many fairly common autoimmune diseases such as MS, rheumatoid arthritis and myasthenia gravis are about 2-3 folds higher in women than in men. Sex hormonal effects are considered to be crucial for the bias. This may also reflect different susceptibility genes between women and men. We therefore considered that it is important to analyze our data separately in women and in men.

Increased frequencies of the KK genotype of ICAM-1 (22/59 vs. 21/68, p = 0.04), allele 2 carriers of IL-1RA (33/49 vs. 23/43, p = 0.043) and the 5G5G genotype of PAI-1 (21/59 vs. 13/68, p = 0.037) were observed in women with MS as compared to the control women. This would suggest tentative associations between ICAM-1, IL-1RA and PAI-1 gene polymorphisms and susceptibility to MS in women. In addition, in logistic regression analyses the pp genotype of ESRI together with DR2 was particularly common in women with MS but not in control women (p < 0.0001),
suggesting that ESRI polymorphism is associated with MS among women with the DR2 allele. A similar finding has also been reported in a Japanese population (Kikuchi et al. 2002). These results suggest that the immunogenetic background of MS may differ between men and women, a conception well supported by the observation that some characteristics of immune regulation are different between the genders. Women develop stronger cell-mediated and humoral responses against stimulus than men, which may be reflected in the increased risk of autoimmune diseases in women (Whitacre 2001). The immunostimulatory property of estrogen may be included in the immunological dimorphism. Estrogens show biphasic dose effects: lower doses facilitate immune responses and higher doses, as those occurring in pregnancy, suppress such responses (Gilmore et al. 1997). Indeed, fluctuations of estrogen during the luteal phase are associated with the number and volume of enhancing lesions in MRI (Pozzilli et al. 1999). In the case of the present findings, estrogen levels in women may be seen to regulate the expression of the potentially susceptibility genes which underlies the expression of MS in women. For example, estradiol may modulate cytokine expression by CD4+ T cells and have the potential to influence the outcome of CD4+ T cell-mediated immune responsiveness (Gilmore et al. 1997). Estrogen receptor is found on many immune cells, but the kind of role estrogens have in the regulation of HLA or other immune system genes is not clear. Our results, together with the above-mentioned Japanese study (Kikuchi et al. 2002), suggest that estrogen receptor polymorphism together with the DR2 allele involved in antigen presentation may modulate the CD4+ T cell response. In a cDNA microarray study treatment with estrogen in EAE down-regulated the expression of TNFα, RANTES and neural cell adhesion molecule and up-regulated that of CTLA-4, TGFβ3, IL-18 and two interferon-γ-induced genes: monocyte chemoattractant protein-1 and vascular cell adhesion molecule (Matejuk et al. 2002).

4. HLA and non-HLA genes in disease severity

There is now evidence that in addition to being related to the susceptibility of MS, genetic factors can modify the course and severity of MS. ApoE seems to be one such (Chapman et al. 2001, Evangelou et al. 1999, Fazekas et al. 2001, Fazekas et al. 2000, Hogh et al. 2000, Schmidt et al. 2002). In addition, RRMS may be more inflammatory than chronic progressive MS (Hemmer et al. 2002), which can be effected by genetic factors, although thus far evidence suggesting this is largely lacking.

HLA\textsubscript{DRB1}-alleles. None of the DRB1/3/4/5-alleles was associated with the severity, course or age at onset of MS, which is in line with the major previous results (Francis et al. 1991, Weinshenker et al. 1998), although a positive finding has also been reported (Runmarker et al. 1994). The present
findings supports the concept that HLA alleles in MS contribute primarily in the establishment of the disease and initial triggering mechanisms rather than influencing disease severity.

**IL-10 gene and disease severity.** None of the studied non-HLA candidate genes was associated with the course of MS. In the case of non-HLA genes and severity of MS, it emerged that the AG genotype of IL-10 is protective against severe MS in all patients (OR=0.32, p = 0.010), the effect being increased over years (10 years; OR=0.33, p = 0.043, 15 years; OR=0.21, p = 0.025 or 20 years; OR=0.14, p = 0.026). This suggest that the IL-10 gene may have an effect on disease severity. Twin and family studies have suggested that genetic factors account for as much as 75 % of interindividual differences in IL-10 production (Westendorp et al. 1997). It seems that IL-10 production is controlled mainly at transcriptional level and genetic polymorphism of the 5' flanking region, for example -1082 polymorphism, may partly explain this variation (Eskdale et al. 1998, Turner et al. 1997). It is therefore possible that IL-10 polymorphism may be functionally associated with the progression of disability in MS. On the other hand, the studied IL-10 polymorphism may be in linkage equilibrium with genetic polymorphisms within the IL-10 gene or with another gene which has a primary effect on MS.

**Complex role of IL-10 on MS.** IL-10 is an important anti-inflammatory cytokine. In the context of MS, it could down-regulate proinflammatory reactions, which are generally believed to contribute to the demyelination seen in MS. In support of this possibility, decreased messenger RNA of IL-10 has been detected in unstimulated peripheral blood mononuclear cells from patients with acute disease (Musette et al. 1996, Rieckmann et al. 1994, van Boxel-Dezaire et al. 1999). However, our finding that a potential intermediate producer genotype of IL-10 –1082 is protective against the severe form of MS while the potential high-producer genotype is not, suggests that intermediate levels of IL-10 are more beneficial in the suppression of the disease than higher levels. Intermediate levels of IL-10 under heterozygous AG genotype may then be neither too great nor too small in the control of inflammatory events in MS. This may mean that the involvement of IL-10 in MS may be complex. Such a possibility could be related to the role of IL-10 in the suppression of inflammation, its role in antibody production and its action on the hypothalamic-pituitary-adrenal axis. The complex role of IL-10 is also suggested by studies showing that mitogen-driven IL-10 secretion varies between increased, decreased or normal in stable RRMS (Balashov et al. 2000, Brod et al. 1997, Trabattoni et al. 2000) and is increased in progressive MS (Balashov et al. 2000).

A clear illustration of the complexity of cytokine networks is to be found studies concerning the role of TNFα in MS. TNFα is considered a proinflammatory cytokine and may be directly involved in the destruction of oligodendrocytes and myelin. However, the neutralization of TNFα
exacerbates MS (The Lenercept Multiple Sclerosis Study Group and the University of British Columbia MS/MRI Analysis Group 1999) and mice lacking TNF develop severe neurological impairment with extensive inflammation and demyelination (Liu et al. 1998). These studies suggest that TNFα may also have anti-inflammatory properties.

**Gene polymorphisms of the IL-10 gene.** The IL-10 gene is known to have many polymorphic sites. In addition to that at position -1082, ten other single nucleotide polymorphisms (SNPs) have been described in the IL-10 promoter (Eskdale and Gallagher 1995, Gibson et al. 2001, Turner et al. 1997). Three of these (-819, -592 and -1082) are in tight linkage disequilibrium. These polymorphisms have been found to present three haplotypes in Caucasian populations (Turner et al. 1997). In addition to these SNP polymorphisms, there are alleles at 2 microsatellite loci formed by variable numbers of dinucleotide CA repeats in the 4 kb and 1.1 kb upstream of the transcription initiation site (Eskdale and Gallagher 1995, Eskdale et al. 1996), designated IL-10.R and IL-10.G polymorphisms, respectively. These microsatellite polymorphisms are likely to be in linkage disequilibrium with the single nucleotide polymorphisms (Eskdale et al. 1999). It is not known which of the numbered polymorphisms in the IL-10 region is the most appropriate for IL-10 secretion. It is interesting that several IL-10.G microsatellite genotypes have been associated with MS progression (Almeras et al. 2002) and the frequency of the IL-10 -2849 polymorphism was different between relapse-onset and progressive MS (de Jong et al. 2002). The polymorphisms associated with MS in previous studies (Almeras et al. 2002, de Jong et al. 2002) and in our present work are located relatively close of each other in the IL-10 promoter region. One aspect of further studies is therefore to take into account possession of particular IL-10 haplotypes and the clinical outcome of MS. This is also important in that haplotype effects may be more relevant than those of a single polymorphism for the IL-10 secretion. Taken together, the present results together with those of two previous studies (Almeras et al. 2002, de Jong et al. 2002) would indicate that upstream polymorphisms of the IL-10 gene may constitute a factor determining the clinical phenotype of MS, suggesting that differential expression of IL-10 can modify the pathogenesis of the disease.

5. **Limitations and future perspectives of the present study**

Any statistical association between an allele and a phenotypic trait is due to one of three situations. First, the finding could be attributable to selection bias; second, the allele might be in linkage disequilibrium with an allele at another locus directly contributing to the phenotype; third, the allele itself might be functional and directly contribute to the phenotype.
**Multiple testing.** Limitations of this study were the relatively small number of subjects recruited and the testing of multiple polymorphisms. We corrected our results for multiple testing when we studied polymorphic regions of chromosomes which comprised the IL-1 region on chromosome 1 and the HLA region on chromosome 6. However, not all polymorphisms studied were included in statistical corrections, since we considered our study explorative the aim being to screen for potential candidates for further studies. Furthermore, we chose polymorphisms of functional significance, since it may be thought that the biological plausibility of the polymorphisms studied might reduce the likelihood of a chance association (Daly and Day 2001).

**Population stratification.** Such case-control studies as ours may be vulnerable to the stratification of the control population, which can also be a source of false-positive findings. An advantage of our study was that we focused on a genetically relatively homogeneous Finnish population characterized by a limited number of founders and national and regional isolation (de la Chapelle 1993). In addition to this genetic homogeneity, one of our control groups was also sex- and age-matched. However, we did not check the geographical origins of our controls and patients. This may be a source of biased associations; genetic regional differences exist also in Finland, since Finns may have different genetic origins (Workman et al. 1976, Kittles et al. 1998). Furthermore, it has been shown that the frequencies of HLA alleles vary between different parts of Finland (Siren et al. 1996). Many solutions have been proposed to the problem of population stratification. One new approach is known as 'genomic control', where the frequency of polymorphisms in other genes unlikely to be associated with the disease are compared between subjects in addition to the polymorphism in the candidate gene of interest, and appropriate adjustment is then made for population stratification (Bacanu et al. 2000). One older and still viable approach is to replicate the study with a new group of patients and controls or use several different control populations (Cardon and Bell 2001). On the other hand, one of the best ways to achieve genetically matched controls is to use family-based controls (Thomson 1995).

The heterogeneity of MS observed in clinical, immunological, MRI and pathological studies may also be seen in the genetics of this disease. Our findings support this, since gender and disease severity emerged as relevant stratifying variables. However, the present results are preliminary, possibly prone to type 1 errors and in need of testing in independent data-sets. One future project of ours is to test our positive results in another Finnish cohort of patients and controls. Based on our results, it would also be interesting to study whether the secretion of IL-10 and PAI-1 differs between patients and controls as well as between different clinical subgroups of patients. Other clear projects in the field of MS genetics are investigations of the role of interactions among genetic...
and environmental / extrinsic factors in susceptibility to and progression of this disease. Exposure to virus infections, especially in childhood, life style and diet are interesting extrinsic parameters to be considered.

6. Future of candidate gene studies in MS

Population-based case-control studies are still relevant in the search for disease-associated genes. It is now realized that very large sample sizes are needed to identify genetic determinants for complex diseases like MS, since most of the susceptibility genes may be of low phenotypic effect in these diseases (Cardon and Bell 2001). Furthermore, study of gene-gene and gene-environmental interactions and different subgroups of MS calls for new statistical methods as well as large sample sizes.

Focus on SNP polymorphisms. One question to arise is, what polymorphisms are worth studying among the increasing data for SNP polymorphisms and others. A map of 1.42 million SNPs distributed throughout the genome has been constructed from the data available in November 2000 (Sachidanandam et al. 2001). It is of note that the HLA locus is a region of high nucleotide diversity, reflecting balancing selection (Sachidanandam et al. 2001). It is rational to choose those polymorphisms which have an effect on gene expression or the function of the protein. SNPs in coding and promoter regions are worth studying, since they are a priori most likely to be of functional significance (Risch 2000). On the other hand, the potential functional significance of any genotype may be evaluated with transgenic animal models and in vitro techniques, using expression and ligand-receptor affinity studies. One approach is to perform haplotype-based association studies, which allow testing of genomic regions for association without requiring discovery of functional variants (Sachidanandam et al. 2001).

New candidate genes in MS. Current candidate gene studies in MS have mainly focused on genes which are closely associated with the T cell function and proinflammatory responses. In the light of our knowledge of the pathogenesis of MS, suggesting that an autoimmune or inflammatory component is essential for the development of the disease (Hemmer et al. 2002), this would still appear to be the right way. However, genes regulating interactions between neuronal, endocrine and immune systems have not been much studied as susceptibility factors in MS. Again, new candidate genes could be chosen as those contributing to the molecular pathways of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system, also including the pathways delivering the effects of sex hormones to the immune system. Another aspect in selecting candidate genes could be related to the neurodegenerative component of MS. Gene expression profiling by cDNA
microarrays from brain tissues or blood cells derived from MS patients may be a tool in the search for new candidate genes for MS. Indeed, cDNA microarrays performed have suggested new molecules, such as leptin, adrenocorticotropic hormone receptor, interleukin-6 and -17 with associated downstream pathways (Lock et al. 2002) and 5-lipoxygenase (Whitney et al. 2001), which may have role in the pathogenesis of MS and points new fields for candidate gene studies in MS. Genome-wide linkage studies are also a tool in the search. Thus far, genome-wide linkage analyses have been a disappointment, but this approach may become more effective, as there is a growing range of SNP maps together with the identification of genes associated with the Human Genome Project. It is clear that genetic studies need to consider that some loci may be involved in the initial pathogenetic events while others influence the development and progression of the disease.

It is believed that some general molecular pathways are involved in the pathophysiology of MS. HLA loci seem to be a crucial factor in susceptibility. A rational base for the case is that repeated findings of associations between specific HLA alleles and a variety of diseases reflects the much higher prior probability of a causal relationship for the HLA loci than for others (Risch 2000). Finally, we may hope that genetic approaches can offer important increments to our understanding of MS and the development of new therapies for this disease.
SUMMARY AND CONCLUSIONS

Multiple sclerosis is a chronic, inflammatory and demyelinating disease of the CNS. Twin, family and population studies have shown that both genetic and environmental factors affect susceptibility to it, although the etiology and pathogenesis of this disease remain unclear. The HLA DR2 haplotype predisposes to MS in Caucasians, suggesting dysregulated immune responses in the expression of MS. Otherwise, genetic factors possibly involved in MS are unknown. In this work the contributions of immune system genes, HLADRB1/3/4/5 alleles, ICAM-1, CTLA-4, ESRI, IL-1 region genes, IL-10, TNFα and PAI-1, were evaluated in MS susceptibility and severity in a genetically relatively homogeneous population of Finns.

The major results and conclusions were:

1. In our case-control study the expected association between HLA DR2 and MS was found. In addition, it emerged that DR1 and DR53 together have a striking protective effect against MS. This observation is novel and suggests that synergistic effects of HLA alleles rather than their individual effects underlie the protective potential.

2. MS susceptibility was not significantly associated with the polymorphisms on ICAM-1, CTLA-4, CCR5, ESRI, TNFα, IL-1 α/β, IL-1RA, IL-10 and PAI-1 genes. However, in subgroup analyses, ICAM-1, ESRI, IL-1RA and PAI-1 genes were significantly or tentatively associated with MS in women but not in men. These findings suggest that the genetic basis of MS may involve differences between women and men. Thus, gender needs to be considered as an important stratifying variable in genetic studies of MS.

3. The HLADRB1/3/4/5-alleles were not significantly associated with disease severity. Of the non-HLA genes studied a functional polymorphism of IL-10 gene affected the severity of MS. This finding would suggest that differential expression of IL-10 can modify the pathogenesis of MS.
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