Linkage and Association Analysis in Multiple Sclerosis

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2001
To my big family
ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system, which is characterized by relapsing or progressive demyelinating plaques in the brain and spinal cord. It is well established that complex genetic factors influence susceptibility to MS. Much work concerning candidate genes and whole genome screens in MS has been done. Many genes or chromosomal regions have been reported to be linked or associated with MS, but the only consistent finding is with the human leukocyte antigen (HLA) region. Here, our aim was to study candidate genes and regions in the Nordic MS population.

We chose linkage and association analysis to investigate a number of non-HLA loci identified in published genome screens to determine whether they play a role for the risk of MS in the Swedish ethnic group and in MS families of similar ethnic origin. Three out of twelve promising chromosomal loci showed association and/or possible linkage in Swedish multiplex families. This suggests the possibility of locating susceptibility genes in 5p, 12q23 and 7ptr-15 (Paper I). The highest NPL score in Nordic affected sibpair families was found in 3p14-13. This region is not only formerly suggested by British and Canadian screens, but also contains the SCA7 gene which may cause spinocerebellar ataxia (SCA), a neurodegenerative disease sharing some clinical features with primary progressive MS. Although no SCA7 gene expansions were observed, we still believe support was obtained for the location of an MS susceptibility gene in this region (Paper II).

Recently, loci of importance for animal models of autoimmune diseases have been identified. We investigated eight chromosomal intervals syntenic to five quantitative trait loci (QTL) in rat collagen induced arthritis (CIA) or oil induced arthritis (OIA) models (Cia2-5, Oia2) in Swedish multiplex families for linkage. 7q34-36, 12p13-12, syntenic to Cia3 and Oia2, showed some indications of importance, in contrast to other regions (2p12, 3p25, 10q11.23, 17q21-25, 19q13, and 22q12-13) (Paper III).

Since MS is thought to be an autoimmune disease mediated by autoreactive T cells directed against myelin antigens, components of the immune system are attractive candidates as MS susceptibility genes. CD40 ligand is an immune regulatory molecule expressed on activated T cells and is found to be expressed in brain sections of MS patients, but not in controls or patients with other neurological diseases. In addition, high mRNA expression of CD40 and CD40 ligand in peripheral blood mononuclear cells (PBMC) of MS patients was recently observed by our group. A CD40 ligand gene (CD40LG) genetic association analysis did not indicate importance of allele variants of CD40LG with mRNA expression in PBMC and no association was found between polymorphisms of CD40LG and MS susceptibility or severity (Paper IV).

Interferon-γ (IFN-γ) is a proinflammatory cytokine thought to have a critical influence in MS pathogenesis. To investigate the possible genetic importance of the IFN-γ gene (IFNG) in MS, we performed a linkage and association study in 100 sib-pair families and 464 severity stratified patients and 266 controls. No linkage or association was found regardless of stratification for sex, severity or DR15. A mRNA expression analysis did not support a reported influence of the 12 CA repeat allele in the first intron of IFNG (Paper V).

In conclusion, six chromosomal regions have received supports for being of importance in MS. Adding more SNP and microsatellite markers for fine mapping these suggestive linkage regions is warranted. For the other studied chromosomal regions and genes, no evidence of linkage or association was found. The access to the entire human genome sequence will greatly facilitate a positional candidate gene analysis.
This thesis is based on the following articles, which will be referred to in the text by Roman numbers:


II. Yamei Dai, Chun Xu, Monica Holmberg, Annette Oturai, Sten Fredrikson, Magnhild Sandberg-Wollheim, Mikko Laaksonen, Anne Spurkland, Frode Vartdal, Lars P Ryder, Per S. Sorensen, Arne Svejgaard and Jan Hillert. Linkage analysis suggests a gene with importance for MS in 3p14-13 (Genes and Immunity, accepted).


IV. Yamei Dai, Thomas Masterman, Wen-Xin Huang, and Jan Hillert Analysis of a CD40 ligand dinucleotide microsatellite in MS (European Journal of Immunogenetics, in press).


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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>APM</td>
<td>affected pedigree member</td>
</tr>
<tr>
<td>APOE</td>
<td>apolipoprotein E</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>CD40LG</td>
<td>cluster of differentiation 40 ligand gene</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CIA</td>
<td>collagen induced arthritis</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EDSS</td>
<td>expanded disability status scale</td>
</tr>
<tr>
<td>EP</td>
<td>evoked potential</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>IDDM</td>
<td>insulin-dependent diabetes mellitus</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IFNG</td>
<td>interferon gamma gene</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>LD</td>
<td>linkage disequilibrium</td>
</tr>
<tr>
<td>LOD</td>
<td>logarithm of odds</td>
</tr>
<tr>
<td>MAG</td>
<td>myelin-associated glycoprotein</td>
</tr>
<tr>
<td>MBP</td>
<td>myelin basic protein</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MOG</td>
<td>myelin oligodendrocyte glycoprotein</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>NPL</td>
<td>nonparametric linkage</td>
</tr>
<tr>
<td>OCB</td>
<td>oligoclonal band</td>
</tr>
<tr>
<td>OIA</td>
<td>oil-induced arthritis</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PLP</td>
<td>proteolipid protein</td>
</tr>
<tr>
<td>PPMS</td>
<td>primary progressive MS</td>
</tr>
<tr>
<td>PRMS</td>
<td>progressive relapsing MS</td>
</tr>
<tr>
<td>RRMS</td>
<td>relapse remitting MS</td>
</tr>
<tr>
<td>QTL</td>
<td>quantitative trait loci</td>
</tr>
<tr>
<td>SCA</td>
<td>spinocerebellar ataxia</td>
</tr>
<tr>
<td>SPMS</td>
<td>secondary progressive MS</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TDT</td>
<td>transmission disequilibrium test</td>
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Clinical aspects in MS

Recent years have seen unprecedented advances in clinical research for multiple sclerosis (MS) including diagnostic techniques and disease-modifying therapies. Although the present thesis mainly deals with genetic aspects of MS, it is relevant to introduce diagnostic criteria and disease severity evaluations used, since correct diagnosis and classification form a basis for genetic studies. Stratification according to clinical subtype or severity could lessen the genetic heterogeneity and facilitate complex disease gene mapping. It is reasonable to anticipate that gene identification will significantly improve our understanding of the pathogenesis of the disease and thereby facilitate the development of effective therapies and finally fulfil the dream of “research serving the patients”.

1. MS diagnostic criteria

MS is a chronic inflammatory disease of the central nervous system (CNS), which is characterized by demyelinating plaques in the brain and spinal cord. Although Charcot, Cruveilhier and others described the clinical features and pathology more than 100 years ago, the etiology of disease is still obscure. There is no definitive diagnostic test for MS, but the emerging modern paraclinical techniques such as magnetic resonance imaging (MRI), oligoclonal bands (OCB) and evoked potentials (EPs) can facilitate diagnosis. The diagnostic criteria used for collecting materials throughout this study are as Poser suggested (Poser et al. 1983) (see table 1), although an international panel on MS diagnosis recently presented revised diagnostic criteria (McDonald et al. 2001).

<table>
<thead>
<tr>
<th>Category</th>
<th>Clinical evidence</th>
<th>Paraclinical evidence</th>
<th>CSF (OCB/IgG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical definite (CD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDMS A1</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>CDMS A2</td>
<td>2</td>
<td>1</td>
<td>and 1</td>
</tr>
<tr>
<td>Laboratory-supported definite (LSD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LSDMS B1</td>
<td>2</td>
<td>1</td>
<td>or 1</td>
</tr>
<tr>
<td>LSDMS B2</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>LSDMS B3</td>
<td>1</td>
<td>1</td>
<td>and 1</td>
</tr>
<tr>
<td>Clinical probable (CP)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPMS C1</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>CPMS C2</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>CPMS C3</td>
<td>1</td>
<td>1</td>
<td>and 1</td>
</tr>
<tr>
<td>Laboratory-supported probable (LSP)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LSPMS D1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Here attacks are defined as symptoms of neurological dysfunction, with or without objective confirmation, lasting more than 24 hours. Clinical evidence of lesions indicates signs of neurological dysfunction demonstrable by neurological examination. Paraclinical or subclinical evidence indicates lesions that are only demonstrable by various tests, such as MRI and EPs, and not by clinical neurological examination. Laboratory support applied here only to examination of cerebral spinal fluid (CSF) for oligoclonal bands and increased production of immunoglobulin G (IgG).

The value of clinical and paraclinical evidence in making a diagnosis of MS is great and the Poser criteria have been the most commonly used for the identification of patients to be included in epidemiology studies or therapeutic trails in recent years.

Epidemiological studies show that at least half of patients with optic neuritis patients later on develop clinically definite MS (Sandberg-Wollheim et al. 1990; Soderstrom et al. 1998). Although clinically isolated syndromes, such as optic neuritis, have the tendency for developing into MS, only definite MS cases were included in our genetic studies.

However, early identification of those patients with optic neuritis who have a high risk of MS, or in fact already have MS, and those who do not have MS or have very small risk of developing MS has become very important, since treatment with recombinant interferon-beta-1a has been shown to be beneficial by reducing the risk for development of CDMS (Jacobs et al. 2000). Therefore, ascertainment of diagnosis of early MS is of clear medical benefit for the patient. In the revised criteria, the focus remains on the objective demonstration of dissemination of lesions in both time and space. These new MS criteria accept the diagnosis of patients with a variety of presentations, including “monosymptomatic” disease suggestive of MS, disease with a typical relapsing-remitting course and disease with insidious progression, without clear attacks and remissions. This reflects an improved understanding of the disease and usefulness of new technology.

2. Clinical category and severity classification

According to the clinical course, MS may be categorized into several different types. A consensus study, based on an international survey of clinicians involved with MS, revealed that there are clear preferences and striking agreement on the meaning of the terms relapsing remitting (RR), primary progressive (PP), and secondary progressive (SP) forms of MS. On the other hand, the authors suggested that the progressive relapsing (PR) MS form deserved a separate definition, as it was not included in the other definitions (Lublin and Reingold 1996). This form has received little subsequent support, and is likely to represent only a small fraction of MS patients. The consensus definitions are as follows:

RRMS: clearly defined disease relapses with full recovery or with sequelae and residual deficit upon recovery, periods between disease relapses characterized by a lack of disease progression.
SPMS: initial RR disease course followed by progression with or without occasional relapses, minor remissions and plateaus may be accepted.

PPMS: insidious onset and disease progression from onset with occasional plateaus and temporary minor improvements allowed.

PRMS: progressive disease from onset, with clear acute relapses, with or without full recovery; periods between relapses characterized by continuing progression.

Primary and secondary progressive MS patients show an increasing disability. A scoring system for disability, the disability status scale, was developed by Kurtzke and later expanded to encompass more subtle changes (Kurtzke 1983). The expanded disability status scale (EDSS) measures MS-related impairment of different functional systems. They are pyramidal functions, cerebellar functions, brainstem functions, sensory functions, bowel/bladder functions, visual functions, cerebral functions and other neurological finding attributable to MS. Accordingly, impairment of MS patients is graded in 20 steps starting from zero (normal neurological examination), increasing to 1 (a single sign only) and then in half-point steps ranging to 10 (death due to MS).

Ebers indicates a median time of 15 years to EDSS 6, 20 years for EDSS 7 and 25 years for EDSS 8 in the relapsing-remitting MS. In the primary progressive group, it was eight year to EDSS 6, 12 year to EDSS 7 and 15 years to EDSS 8 (Ebers 2000).

Concerning severity of disability, definitions of “benign” and “malignant” MS have also been suggested (Lublin and Reingold 1996). Here, benign MS means disease in which the patient remains fully functional in all neurologic systems after 15 year of disease onset. Malignant MS was defined as disease with a rapidly progressive course, leading to significant disability in multiple neurologic systems or death in a relative short time after disease onset.

Usually, “benign MS” is considered a subcategory of RRMS. A difference between RRMS and benign MS would be that RRMS attacks are more frequent and some patients have a stepwise increase in neurological deficit.

3. Clinical features

During the course of the disease, MS patients exhibit a wide range of clinical symptoms and signs, few of which is considered particularly specific for the disease. Long-term outcome, in terms of disease severity, is however strikingly uniform (Ebers 2000).

Symptoms and signs of MS patients are thus variable, depend on the part of the CNS affected. Some tend to appear early in disease course, some later and many vary between patients. Weinshenker (Weinshenker et al. 1989) and colleagues demonstrated that sensory impairment was the most common symptom at initial
presentation, followed by optic neuritis, insidious motor functional impairment, limb ataxia, diplopia and/or vertigo and acute motor functional loss. Other symptoms and signs such as abnormal reflexes, impairment of bowel and bladder control and sexual dysfunction also could be found. Memory impairment and similar cognitive dysfunction and affective disorders are more frequent among patients with MS compared with general populations (Miller 1998).

Immunogenetic studies of human leukocyte antigen (HLA) genes have suggested that there maybe two distinct subtypes of MS: Asian-type and Western-type. In a recent study, Kira et al (Kira et al. 1996) reported the clinical differences between Western and Asian types of MS based on the natural history of the disease and magnetic resonance imaging scanning of the brain and spinal cord of a group of Japanese patients. These patients, residing in Kyushu, were classified as having “Western type” or “Asian type” MS. Asian type MS was characterized by a relapsing-remitting course and a selective involvement of the optic nerve and spinal cord, a disease pattern that is well-known to occur in some Asian populations. In contrast, patients with “Western type” MS had evidence of more disseminated central nervous system disease. Since Western type MS patients showed a DR2 association, while Asian type MS patients did not, he suggested the presence of two etiologically distinct diseases in Asians.

4. Treatment

MS has been long been considered as an untreatable disease, and a number of pharmacological agents failed to show an effect, including corticosteroids, azathioprine, cyclophosphamide and cyclosporin A. Although interferon-β (IFN-β) and glatiramer acetate have a documented effect and intravenous immunoglobulins (IVIG) and mitoxantron are likely to share this property, all these drugs are only partially effective and not all patients respond well or tolerate side-effects (Amor et al. 1997; Rolak 2001). Choosing specific treatment for a given patient and using specific drugs in different disease stages could potentially help getting better results. Genetic determination can be hypothesized to be responsible for some of the diverse therapeutic effects. Thus, identification of genetic polymorphism influencing drug metabolism may significantly facilitate the development of effective therapies.

Basic researches in MS

Since MS was first described, it has become intensively studied with regard to epidemiology, pathology, immunology and genetics, but still many central questions await satisfying answers. Identification of key pathogenic mechanisms in MS would provide a basis for the development of causal therapeutic strategies applicable to all MS patients. But since a variety of different immunological mechanisms may lead to demyelination and since high inter-individual although low intra-individual variability of pathologic lesions has been found, a number of different pathogenic mechanisms may exist. Such heterogeneity of possible pathogenic mechanisms is likely to be reflected in profound clinical and pathological characteristics, and may well be based on different
genetic susceptibility factors.

1. Epidemiological features

Epidemiological studies have provided clear evidence for the importance of genetic factors for susceptibility and environmental factor may also play an important role.

Onset of MS mainly occurs in 20-50 year-old individuals, more often females than males. MS has an uneven geographical distribution. Regions close to the equator have low prevalence, while the prevalence increases with the increase of latitude, probably independent of genetic and racial factors (Kurtzke 1977; Skegg 1991).

Migration studies (Alter et al. 1966; Dean and Elian 1997; Dean and Kurtzke 1971) and a possible MS epidemic in the Faroe island (Kurtzke and Hyllested 1979) support an environmental influence, however, migration studies are methodologically difficult and data from these studies have been challenged in recent years (Paty and Ebers 1998). Various viral infections, chemical agents assuming influencing MS have been carry out, but there is insufficient evidence to draw any definite conclusions concerning any of the viruses or other agents so far proposed (Boman et al. 2000; Casetta and Granieri 2000; Sibley et al. 1985; Sriram et al. 1999).

About 20% of MS patients have one or more affected relatives (Ebers et al. 1995; Sadovnick et al. 1988). The 30% concordance rate among monozygotic twins, a 10-fold increase over that of dizygotic twins or first-degree relatives, indicates a genetic effect (Ebers et al. 1986; Ebers et al. 1995). While individuals sharing genes (i.e. monozygotic twins) also have a 70% discordance rate this may be indicative of an environmental effect. However, yet another factor of possible importance is that of random chance, “bad luck”.

One measure of familial aggregation is the \( \lambda \) statistic, defined as the ratio of the lifetime risk in relatives of affected individuals (K relative) versus the population prevalence- (K) of the disease (\( \lambda = K_r/K \)) (Risch 1992). When compared with the lifetime risk of Northern European Caucasians (~1:300), we could see the higher increased disease risk in the tighter biological relatives of MS patients (see table 2).

<table>
<thead>
<tr>
<th>( \lambda )</th>
<th>Risk Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>100-190</td>
<td>(identical twins)</td>
<td>(Mumford et al. 1994; Sadovnick et al. 1993)</td>
</tr>
<tr>
<td>60</td>
<td>(offspring of two affected parents)</td>
<td>(Robertson et al. 1997)</td>
</tr>
<tr>
<td>13</td>
<td>(dizygotic twins and full sibs)</td>
<td>(Robertson et al. 1996)</td>
</tr>
<tr>
<td>7</td>
<td>(half-sibs)</td>
<td>(Sadovnick et al. 1996)</td>
</tr>
<tr>
<td>7</td>
<td>(other first degree relatives)</td>
<td>(Robertson et al. 1996)</td>
</tr>
<tr>
<td>5.5</td>
<td>(single affected parent)</td>
<td>(Robertson et al. 1996)</td>
</tr>
<tr>
<td>3.5</td>
<td>(second degree relatives)</td>
<td>(Robertson et al. 1996)</td>
</tr>
<tr>
<td>1</td>
<td>(adoptees)</td>
<td>(Ebers et al. 1995)</td>
</tr>
</tbody>
</table>
On the basis of results from family, twin, and adoption studies, it now clearly indicates that genetic factors play a major role in MS. In addition, racial clustering of MS cases, resistant ethnic groups sometimes reside in high-risk regions also supports a genetic effect (Gronning and Mellgren 1985; Kalman et al. 1991; Skegg et al. 1987).

In all, risk of MS seems to be influenced by both genetic and environmental factors. Consideration of the interaction between genes and environment may well facilitate gene identification.

**2. Distinct pathologic pattern indicates different immunopathogenesis**

MS affects principally the white matter of the CNS. The gross pathology is characterized by plaques that appear as scattered, irregularly shaped, usually translucent gray area, about 1-15 mm in diameter. Extension of lesions into the surrounding white matter along blood vessels crossing the plaque margin (Dawson’s fingers) is common. Sites of predilection in the brain include the white matter around the ventricles and the corpus callosum. Lesions are also frequent in the optic nerves and cervical spinal cord. Progressive brain and spinal cord atrophy is sometimes demonstrable already in early and clinically mild MS (Liu et al. 1999; Stevenson et al. 1998).

Histopathologically, acute (with inflammation, hypercellular regions) and chronic (without inflammation, hypocellular regions) MS plaques are quite different. Acute MS plaques are characterized by multifocal perivenular infiltration of the white matter by lymphocytes and monocytes/macrophages in the CNS and selective destruction of myelin and myelin-forming oligodendrocytes that at least initially spares the axons. Chronic MS plaques are sharply circumscribed and hypocellular with no evidence of active myelin breakdown. Fibrillary gliosis is prominent and axonal density is often markedly reduced. Regardless of which kind of pathogenic pathway and clinical course, the final pathology is usually considered to be similar. (Poser 2001) While in active MS lesions, different patterns of histopathology were observed.

Recently, a group of Austrian, American and German scientists (Lucchinetti et al. 1998) described four patterns of demyelination of suggested different pathogenic nature. This concept was based on a broad spectrum of immunological markers, neurobiological markers testing myelin protein loss, the topography and extension of plaques, pattern of oligodendrocyte destruction, and evidence of complement activation. Two patterns (I and II) showed close similarities to T-cell-mediated or T-cell plus antibody-mediated experimental autoimmune encephalomyelitis (EAE), respectively. The other patterns (III and IV) were highly suggestive of a primary oligodendrocyte dystrophy, reminiscent of virus- or toxin-induced demyelination rather than autoimmunity.

This heterogeneity in pathology may well reflect different pathogenesis. Whereas this may seem logical, Poser (Poser 2001) has cast doubt on the differential diagnosis between MS and ADEM in this material since the pathologic material acquired from needle biopsy in some of these studies is difficult to settle. Actually the classic clinical
pathological pattern of chronic MS represents only one member of a family of closely related inflammatory demyelinating leukoencephalitides that include acute MS (Marburg variant), Balo’s concentric sclerosis, neuromyelitis optica (Devic’s disease), and acute disseminated encephalomyelitis (ADEM). Although the clinical and pathological characteristics of these diseases are diverse, the presence of transitional forms suggests a spectrum of inflammatory diseases that may share a pathogenic relationship (Lucchinetti et al. 1998).

Several lines of evidence indicate MS is a T cell mediated immune disease. First, appearance of immune cells and immune related compounds indicate the involvement of the immune system, most probably deviated autoimmune system. There are increased numbers of myelin antigen-reactive T cells present in the peripheral blood and CSF of MS patients (Sun et al. 1991a; Sun et al. 1991b). T and B lymphocytes present in MS lesions or in the cerebrospinal fluid of patients, show signs of activation; i.e., the classic IgG oligoclonal bands of the cerebrospinal fluid (activation of B lymphocytes) and the presence of activation markers on the surface of the T-cells.

Second, the well-established association and linkage of HLA class II alleles and haplotypes suggest an importance of the immune system, since the major histocompatibility complex (MHC) contains the strongest immune response genes we know of.

Third, immune modulation exerted by IFN-β has a positive effect on MS reported by the IFNB Multiple sclerosis study group (1995), whereas measures that activate the immune system (i.e. IFN-γ) seem to have a negative effect (Panitch et al. 1987).

Finally, passive transfer of myelin specific T cells (Pettinelli and McFarlin 1981) and myelin oligodendrocyte protein (MOG)-specific antibodies (Linnington et al. 1988) to animals could induce EAE, an autoimmune MS like disease usually analyzed in rodents. EAE can be induced by several myelin components: myelin basic protein (MBP), proteolipid protein (PLP), MOG and myelin-associated protein (MAG) most often together with an adjuvant.

Many MS schematic diagrams and figures are drawn, in attempts to include both etiology and possible immunopathogenesis (Hohlfeld 1997; Noseworthy 1999). Figure 1 shows an autoimmune inflammatory reaction mediated by helper T-cells that have passed blood brain barrier (BBB) and cause myelin damage.
3. Review of genetic research in MS

Genetic epidemiology has strongly suggested the involvement of genetic determinants in MS. It is also clear that complex genetic factors influence susceptibility to MS, i.e. that several genes are acting, alone or in additive or multiplicative way. However, how may one identify the MS susceptibility genes among the at least 30,000 genes that humans possess? The following approaches have been used.

3.1 Candidate gene studies

Genetic analysis in MS has traditionally focused on candidate polymorphic genes. They are often suggested by the pathophysiologic and clinical features of the disease. Since MS is thought to be an autoimmune disease mediated by autoreactive T cells directed against myelin antigens, the various components of immune system such as HLA, T cell receptor (TCR), Ig, probable autoantigens like MBP, MOG, PLP, various cytokines, chemokines and their receptors etc. are attractive candidates for MS susceptibility gene studies.

The HLA complex, which is located on chromosome 6p21, is the best-studied region in MS as in most other autoimmune disorders. In 1972 Jersild first reported that MS is
associated with HLA-A3, B7 and Dw2 (Jersild et al. 1973; Jersild et al. 1972). With the advances in HLA typing techniques, from cellular typing to serology and later to the DNA level, full identification of HLA polymorphism is now possible. Thus, we now know that cellularly defined specificity Dw2, which has a confirmed role in MS corresponds to DR15 and DQ6 in serologic nomenclature and to the haplotype DRB1*1501, DRB5*0101, DQA1*0102, DQB1*0602 by genomic nomenclature (Hillert 1994). Association and linkage with HLA have been consistently confirmed in MS. Although occasional reports lacked significant evidence for either association or linkage, the overall picture is clear (Cullen et al. 1991; Ebers 1982; Fogdell et al. 1997; Francis et al. 1991; Gogolin et al. 1989; Hillert et al. 1994; Hillert and Olerup 1993; Marrosu et al. 1992; Spurkland et al. 1991; Stewart et al. 1981). The occasional lack of replication may arise from low power due to small sample size, random variation, or possible ethnic group effects.

TCR recognize antigen presented by MHC molecules and are of two isoforms: TCRαβ, TCRγδ. The α,δ chain genes are located together on chromosome 14 and the β and γ chain genes are separately located on chromosome 7. Linkage and association studies concerning these gene polymorphisms have been extensive and results varying. In an early study, authors reported a significant increase of an α chain allele in DR15+ patients compared with controls, but other investigators found no evidence of association or linkage to the α chain genes (Eoli et al. 1994; Hashimoto et al. 1992; Hillert et al. 1992; Martell et al. 1987; Oksenber et al. 1989). The TCR β gene cluster has been extensively investigated as a candidate gene as well. Thus indications of linkage have been reported (Haines et al. 1996; Seboun et al. 1989; Wood et al. 1995b), but also failed to be observed (Lynch et al. 1991; Sawcer et al. 1996). Likewise, some studies have shown associations (Beall et al. 1989; Charmley et al. 1991; Epplen et al. 1997; Hockertz et al. 1998; Martinez-Naves et al. 1993), whereas others have not (Droogan et al. 1996; Fugger et al. 1990; Hillert et al. 1991; Vandevyver et al. 1994; Wei et al. 1995).

Increase intrathecal Ig production is present in a very high frequency in MS patients and antibodies against CNS specific antigens have also been found (Link et al. 1989). The gene cluster coding for the Ig heavy chain is located on chromosome 14q. There are reports showing evidence of association (Gaiser et al. 1987; Walter et al. 1991) and week linkage (Wood et al. 1995a), but negative results were also observed (Ebers et al. 1996; Hillert 1993; Ligers et al. 1997).

Many kinds of protein and lipids contribute in the formation of myelin. Myelin proteins are therefore considered as possible autoantigens, and the myelin associated enzyme 2’-3’-cyclic nucleotide-3’-phosphodiesterase (CNP:ase) has been suspected to be involved in the inflammation process. The genes encoding MBP, PLP, MOG, MAG, and CNP:ase are located on 18q22-qtr, Xq21-22, 6p22-21.3, and 19q13,17q21 respectively. There have been several efforts to reveal a genetic importance of MBP, PLP, MOG, MAG, CNP:ase gene polymorphisms in MS by association analysis and linkage (Boylan et al. 1990; He et al. 1998; Price et al. 1997; Roth et al. 1995; Tienari et al. 1992). Except the positive linkage and association data from Finland, mostly negative results have been
Cytotoxic T lymphocyte antigen 4 (CTLA-4) is an important co-stimulatory molecule expressed on the surface of activated T cells, which can down-regulate T cell activation (Karandikar et al. 1996). The gene encoding CTLA-4 is located on chromosome 2q33. Positive associations with MS have been found in our group as well as by others (Fukazawa et al. 1999b; Harbo et al. 1999; Ligers et al. 1999), and CTLA-4 gene expression is indeed influenced by promoter and exon 1 polymorphisms (Ligers et al. 2001).

Genes encoding cytokines, chemokines and their receptors such as TNFα, β, IFN-α, β, γ, TGF-β, IL-1a (interleukin 1a), IL-2, IL-4, IL-10, IL-12, MCP-3, CCR5 and their available polymorphic receptors have also been investigated in MS (Crusius et al. 1995; Epplen et al. 1997; Fiten et al. 1999; Goris et al. 1999; He et al. 1998; McDonnell et al. 2000; Mertens et al. 1998; Sudomoina et al. 1995; Vandenbroeck et al. 1999; Vandenbroeck et al. 1997; Vandenbroeck et al. 1998). No convincing associations have so far been found.

By screening candidate loci from peaks of possible linkage in previous screens, Chataway et al found evidence for the importance of the myeloperoxidase gene in MS (Chataway et al. 1999a). In the same way, a number of genes including complement factors 6 and 7 were considered less interesting or even excluded (Chataway et al. 1999b).

Apolipoproteins are a group of proteins responsible for transport of lipids. Apolipoprotein E (APOE), APOB, APOC-II and APOH have been investigated (Barcellos et al. 1997; Chataway et al. 1999a; Gervais et al. 1998; Weatherby et al. 2000; Zouali et al. 1999), APOE and APOC-II have received support for a role in MS in the studies by Barcellos and Zouali.

Vitamin D receptor is considered as a relevant candidate gene as well. A Japanese group has reported a positive association with a Vitamin D receptor gene polymorphism in MS (Fukazawa et al. 1999a; Niino et al. 2000). In 1992, Harding et al reported the occurrence of an MS-like illness in women who have a Leber’s hereditary optic neuropathy with mitochondrial mutation (Harding et al. 1992). These patients have the clinical manifestations of MS and typical magnetic resonance changes and oligoclonal bands. Therefore several other groups search the mitochondrial mutations in unrelated MS patients but without positive finding (Kalman et al. 1995; Kellar-Wood et al. 1994; Nishimura et al. 1995). Anyhow, this observation is of importance since it indicates that an MS-like syndrome can occur on various genetic backgrounds.

3.2 Whole genome screens
Using markers throughout the genome to find susceptibility gene is an appealing method for mapping disease genes also for complex diseases.
Four genome screens in MS have so far been published (Ebers et al. 1996; Haines et al. 1996; Kuokkanen et al. 1997; Sawcer et al. 1996). None of these studies identified a single locus with formally significant linkage, but each study indicated the possible importance of a number of chromosomal regions. Although, over 60 such genomic regions were picked out depending on the statistical cut-off chosen, naturally, most of such loci are expected to be false positive. The only consistent regions in these screens were 6p21 and, possibly, the centromeric region of chromosome 5. In a meta-analysis of these four genome screens, Wise identified five regions with a p value less than 0.05 (Wise et al. 1999). A more recent meta-analysis performed by the original investigators (Ebers et al. 1996; Haines et al. 1996; Sawcer et al. 1996) revealed eight chromosomal regions with nonparametric linkage (NPL) scores greater than 2.0 (2001)(see table 3).

Table 3 Chromosome regions of interest revealed by genome screens and meta-analyses

<table>
<thead>
<tr>
<th>UK genome screen:</th>
<th>1p/cen, 2cen, 3p/cen, 4q, 5cen, 6p/q, 7p, 11p, 12p, 14q, 17p/q, 19q, 20p, 21p, 22q, Xcen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canadian genome screen</td>
<td>1p, 2p/q, 3p/q, 4p/q, 5p/q, 6q, 7p/q, 10q, 11q, 14q, 15q, 16q, 18p/q, 19q, Xp/q</td>
</tr>
<tr>
<td>American/France genomic screen</td>
<td>2p, 3q, 4q, 5q, 6q, 6q, 7q, 9p, 9q, 10q, 11p, 12q, 13q, 16p, 18q, 19q</td>
</tr>
<tr>
<td>Finnish genome screen</td>
<td>2q, 3q, 4cen, 5p, 6p, 10q, 11tel, 17q, 18tel, 19tel</td>
</tr>
<tr>
<td>Meta-analysis from Wise</td>
<td>2p, 5p, 6p, 17q, 19q</td>
</tr>
<tr>
<td>Meta-analysis from transatlantic cooperation</td>
<td>3p, 5q, 6p, 6q, 12p, 16p, 17q11, 17q22</td>
</tr>
</tbody>
</table>

Follow up studies concerning genomic regions of interest had been published (Chataway et al. 1998; Chataway et al. 1999a; D’Alfonso et al. 1999; Larsen et al. 2000; Oturai et al. 1999; Seboun et al. 1999; Xu et al. 1999). In the Nordic population, we have participated in getting some support for the loci in 5p, 6p, 7p, 12q, 17q (Larsen et al. 2000; Oturai et al. 1999; Xu et al. 1999).

The findings in genome screens of several potentially interesting chromosomal regions but failure to detect one major gene, supports the notion that MS is indeed a polygenic disease where many genes are involved, each having a minor effect. These observations in MS have followed the development in several other comparable disorders such as diabetes mellitus.

3.3 Study chromosomal regions syntenic to animal model diseases

Loci of importance for animal models of MS and other autoimmune diseases have been identified and rough comparative map between rat, mouse and human already developed (Dahlman et al. 1998; Lorentzen et al. 1995) (http://www.ncbi.nlm.nih.gov/Homology). As meta analysis shows there are homologous genomic regions associated with different autoimmune diseases in both mice, rats and humans (Becker et al. 1998; Encinas and Kuchroo 2000; Griffiths et al. 1999; Kawahito et al. 1998), therefore study chromosomal regions syntenic to animal models could help identification of genes...
predisposing to human diseases. Though syntenic region study, Kuokkanen and his colleagues revealed 5p is of important for MS susceptibility (Kuokkanen et al. 1996).

**Characteristics of mapping complex disease genes**

Complex disease has a genetic component that is not strictly Mendelian for example dominant, recessive, or X-linked. They are characterized by the risk to relatives of an affected individual that is greater than the incidence of the trait in the population. This kind of disease, such as insulin dependent diabetes mellitus (IDDM), MS or cardiovascular diseases has complex etiologies comprising both genetic predisposition and environmental influences (Haines and Pericak-Vance 1998).

Many genes may confer susceptibility to the disease development or even different genes may be involved in different disease stages. Such genes are likely to have modest effects on the risk of developing disease; their alleles are likely to be common in the population and frequencies may differ between different populations. Therefore, when mapping genetically complex disease, we are not searching for one or few rare mutations in a single gene that cause a severe disruption of a single gene product and a devastating disease as a consequence. We are rather searching for alterations in more than one gene that alone or in concert either increase or decrease the risk of developing a trait. In summary, genetic alteration underlying complex disease may not be rare at all, but rather be common polymorphisms.

**Linkage and association analysis used in mapping disease genes**

The importance of a locus or a gene for a disease can be investigated by linkage or association, which is composed of different methods (see table 4).

Genetic linkage usually refers to the tendency of genes or other DNA sequences or traits such as disease to be inherited together as a consequence of their physical proximity on a single chromosome (Terwilliger and Ott 1994).

Linkage analyses are analytical methods used for testing linkage. In its simplest form, linkage analysis consists of counting recombinants and nonrecombinants, estimating the recombination fraction (theta) and testing whether this fraction is significantly less than 0.5. This is the so-called direct method of linkage analysis.

However, in most human pedigrees, when the gamete phase is unknown, when penetrance is incomplete, and when the size of families is limited or other factors cloud the direct view of genotypes through phenotypes, it is not possible to identify recombinants unambiguously and count them. It is possible, however, to calculate the overall likelihood of the pedigree, on the alternative assumptions that the loci are linked or not linked. The ratio of these two likelihoods gives the odds of linkage, and the logarithm of the odd ratio is the lod. Presence of linkage is accepted if the lod exceeds a
certain threshold, T, traditionally T=3. On the other hand, a common exclusion threshold is T=-2. This method is based on a pre-specified genetic model and therefore referred to as a parametric method. But in complex disease gene mapping, it is difficult to specify a correct genetic model, detailing the mode of inheritance, gene frequencies and penetrance of each genotype. If the mode of inheritance is wrong, it could lead to false positive and negative results. Thus, non-parametric analyses are to be preferred.

Table 4. A principle comparison of linkage and association analyses used in the present study

<table>
<thead>
<tr>
<th>Methods</th>
<th>Linkage analysis (Larger Pedigrees)</th>
<th>Linkage analysis (sibpairs, relative pairs)</th>
<th>Transmission disequilibrium test</th>
<th>Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Also commonly referred to as</td>
<td>LOD-score analyses Model-based methods</td>
<td>Non-parametric methods Allele-sharing methods Model-free methods</td>
<td>Family based association analysis</td>
<td>Population based case-control analysis</td>
</tr>
<tr>
<td>Goal</td>
<td>To identify the probable location of a disease gene</td>
<td>To determine whether a candidate allele is associated or in linkage disequilibrium with the disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Material Type</td>
<td>Large families contains many affected relatives</td>
<td>Many sibpairs or relative pairs</td>
<td>Affected probands and heterozygous parents</td>
<td>Affected individuals and controls</td>
</tr>
<tr>
<td>Usage, advantage and disadvantage</td>
<td>Could be used for analysis of a specific gene or the whole genome</td>
<td>In candidate gene study.</td>
<td>TDT can circumvent the problem of poorly matched control group by using information on parental genotype to create an internal control for comparison</td>
<td>Being sensitive, able to detect small genetic effect, but also prone to generating false-positive associations. Lack of confirmation in many cases</td>
</tr>
</tbody>
</table>

Non-parametric linkage analyses also called model-free linkage analyses. These methods usually look for alleles or chromosomal segments that are shared by affected individuals. By random segregation, sib pairs expect to share 0, 1, 2 parental hyplotypes $\frac{1}{4}$, $\frac{1}{2}$, $\frac{3}{4}$ of the time. But in disease it will cause deviation of this proportion in disease linked loci. Sibpair analysis is a form of genetic analysis in which markers
are tested for linkage to a disease or phenotypic trait by measuring high hyplotype sharing in a panel of affected sib pairs. (Strachan and Read 1996) Shared segment methods can be used within nuclear families (sib pair analysis), within known extended families (affected pedigree member analysis, see method section).

There are two types of association studies. Population based case-control studies and family based association studies. At the population level association analysis can be done in a case control manner, by comparing the frequency of marker alleles between unrelated affected and unaffected individuals. At the family level, association analysis can be performed by comparing the frequencies of alleles transmitted to an affected individual to the alleles not transmitted. These methods could determine whether a candidate allele is associated or in linkage disequilibrium (LD) with the disease. When alleles at two distinct loci occur in gametes more frequently than expected given the known allele frequencies and recombination fraction between the two loci, the alleles are said to be in LD. Evidence for LD can be helpful in mapping disease genes because it suggests that the two alleles may be very close to one another (Haines and Pericak-Vance 1998).

**Major steps in carrying out gene mapping in complex disease**

The approximate chromosomal location of genetic factors by linkage is the first step in positional cloning of disease genes. Linkage and positional cloning have had great success in leading to the identification of the genes for many Mendelian diseases in the past twenty years but it seems much more difficult for common diseases without clear Mendelian inheritance patterns. In complex disease gene mapping, several steps are involved and this may deserve more careful consideration than classic positional cloning of Mendelian traits. The following are the major steps for carrying out gene mapping in complex disease.

1. **Define phenotype, determine whether the trait has genetic component**

Segregation analysis, relative risk estimations, twin, adoption studies or heritability analysis determine whether a trait has a genetic component. A consensus diagnostic scheme must be established *à priori* and must possess consistency and stringency since mapping of complex diseases usually require large data sets of family data collected across multiple centers. It is crucial that the same diagnostic criteria are used by all participating centers.

2. **Develop experimental design**

The exact approach to the mapping process should be outlined as completely as possible before the project gets under way. It includes decisions of what kinds of material, markers and analysis to use. The large families with known model of inheritance and many affected individuals, which classical linkage analysis relied on, typically do not occur in complex diseases. In complex disease, the model of inheritance is unknown, therefore nonparametric linkage analyses based on affected sib-pairs or other relative
pairs are likely to be suitable. Simulation analysis performed to determine appropriate sample size is also needed before starting experiment. If enough families are unlikely to be collected, the analysis needs to be based on other materials, such as large numbers of cases and controls or sporadic patients with available parents for transmission analysis.

3. Identify families and collect biological samples
This work should be done by clinicians with experience of the disease in focus. Ascertainment of families is performed according to identical diagnostic criteria after which collection of biological samples may be performed.

4. Genotype samples with markers
The methods to detect genotype are numerous. Factors to consider in choosing a method include the number of samples that will be analyzed, cost and whether radioactivity is an option. For microsatellite polymorphism fluorescence may be the fastest method as well as generally available. Microsatellites are typical markers to be used in the mapping stage of the analysis. However, when it comes to analysis of specific genes, it may be better to base the analysis on functionally important polymorphisms such as base substitutions.

5. Analysis of the genotyping data
Many kinds of statistical analytical methods may be used. See below in the statistical analysis section in “Materials and methods”. The choice of method may depend on the stage of the mapping process. For example, sib-pair analysis may be used in the initial genomic screening, but the detailed analysis of specific genomic regions may use association studies.

6. Identification, re-testing and fine-mapping of genetic regions of interest
Gene mapping may be more difficult in complex disease than in monogenic diseases. Since complex diseases often involve more than one gene, each gene has only a minor effect, and since the risk of genetic heterogeneity is great, it may be difficult to get significant linkage results in the initial genomic screen. In first stage genomic screen, the goal is find out a small subset of genomic regions that might harbor susceptibility genes. The retest process is to eliminate any false positive results and to confirm any true positives by adding more markers and more samples. Once one or a few regions have been confirmed as harboring susceptibility genes, the goal is to narrow that region as much as possible.

7. Identify disease gene by positional candidate gene approach
After the release of entire human genome sequence, gene mapping is likely to become easier. Once a region has been sufficiently narrowed down, a positional candidate gene approach to find disease gene can start. However, since the kinds of polymorphisms
involved are likely to be minor changes influencing gene expression or functional properties of proteins, it may be very difficult to prove the importance of a particular gene among several genes. This stage is likely to include functional analysis, which therefore has to be adapted to the specific situation.

8. Define complex genetic interactions
This is in itself a poorly explored area where methods need to be established. Combining epidemiological and genetic approaches could be help in unravel the complexities of gene–gene and gene environment interactions.
AIMS OF THE STUDY

To investigate candidate loci indicated to be of possible importance in previous genome screens (papers I and II).

To study chromosomal regions syntenic to those identified as important in autoimmune animal models (Paper III).

To study \textit{CD40L}G and \textit{IFNG} polymorphism effects on disease susceptibility, disease severity and gene expression.
MATERIALS AND METHODS

Materials

1. Families

In collaboration between nine centers in four Nordic countries, more than 100 families with affected sibling pairs with MS have been identified. Families were ascertained by clinicians at the participating centers. Clinical diagnosis was defined according to Poser’s criteria (Poser et al. 1983). Positive family histories were investigated by direct contact with other family members, requests for medical records and when indicated by clinical examination, laboratory testing or paraclinical studies (OCB/IgG, MRI, EPs). All affected MS patients were examined or had their medical records reviewed by one of collaborators. Only definite MS patients were considered as affected.

Besides sib-pair families, we also included 24 Swedish multiplex families, which contained affected parent-child pairs, affected aunt (uncle)-nephew (niece) pairs, affected grandparent-grandchild pairs, and affected half-sibpairs. These families were used for linkage analysis.

A small number of singleton families were also collected for transmission analysis.

2. Sporadic patients

Sporadic cases and ethnically matched healthy controls were collected for association studies. These patients were stratified according to disease severity into a severe group, a benign group and an intermediate group. We arbitrarily defined an EDSS score of 3.0 or less despite duration of at least 10 years as benign MS, and an EDSS score of 6.0 within eight years of disease onset as severe MS. The purpose of applying EDSS to group the patients was to enable comparisons of extreme phenotypes in order to identify phenotypic determinants.

Methods

1. Genotyping of microsatellite polymorphisms

Polymerase chain reaction (PCR) is a cell-free method of DNA amplification. It allows amplification of specific DNA segments in presence of a DNA template, Taq polymerase, dNTPs, PCR buffer and the primer pairs. In our study, fluorescently labeled microsatellite markers were used. Forward primers for each primer pair were labeled with 5’-FAM, HEX, or TET phosphoramidite (Cyber gene, Sweden). PCR was performed in 7ul PCR reaction volumes containing 0.2 uM of each primer, 0.12 units of Taq polymerase (Phamacia), 1.5 mM of MgCl₂, 0.2mM of dNTP and 20ng of genomic DNA.
Most common PCR conditions consisted of denature at 95°C for 4 minutes, 95°C 30 seconds, 55°C 30 seconds, 72°C 40 seconds for 30 cycle, then followed by 72°C 6 minutes extension. Only a few markers required different annealing temperatures and different cycles. After PCR amplification, PCR products were diluted and combined with internal lane size standard. The sizes of the PCR products were detected using Genescan software (GENESCAN 2.0.2) on 377 ABI DNA sequencer. Genotypes of the markers for each individual were analyzed using Genotyper software (GENOTYPER 1.1).

2. Detection of mRNA expression in PBMC by competitive RT-PCR

Blood samples were taken from MS patients and healthy controls for quantification of mRNA expression. PBMC were isolated within two hours of sampling by density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway) using a standard protocol. Separated PBMC were divided into aliquots of 5x10⁶ cells and precipitated by centrifugation. The cell pellets were immediately frozen by liquid nitrogen and stored at -70°C until further use. Total RNA was isolated from 5x10⁶ cells following a protocol using guanidine thiocyanate and phenolchloroform extraction with modification of two extractions by phenol-chloroform at PH 4.0 to minimize contamination of genomic DNA. The RNA product was resuspended in diethyl paracarbonate (DEPC) treated water to a final concentration of approximately 3.5 ug/32ul. Complementary DNA (cDNA) was synthesized using oligo d(T)₁₆ as primer according to the instructions of the manufacturers of the reagents (Perkin-Elmer) with slight modification of extending the time of reverse transcription reaction to 45 min. The cDNA products later on were used together with designed competitor here named as “internal standard” (IS) as template. Therefore the competitive PCR reaction system contained same primer sequence motif but a different length template, so two different length peaks were detected on ABI 377 DNA sequencer. Through the relative proportion of competitive PCR products (area covered by the peaks), using the standard curve, the amount of cDNA could be estimated. To compensate the relative differences among samples, in the integrity of the individual of RNA samples and the variants in reverse transcription, β-actin cDNA was also amplified and quantified for each test sample. The estimated amount of target cDNA/mRNA was divided by the amount of house keeping gene β-actin in each sample before comparison (Huang et al. 1999).

Statistical analyses

Parametric and nonparametric linkage analyses were used in analysis of genotyping data in families.

1. Parametric linkage analysis

A Parametric method was used in Paper I together with nonparametric linkage
Two-point linkage analysis was performed with MLINK version 5.10 of the LINKAGE package (Lathrop et al. 1984). Equal recombination fractions were used for males and females. The age dependent penetrance values were 0.7, 0.35, 0.1 and the disease gene frequency was 0.001.

Since the mode of inheritance for MS remains unclear and parametric linkage analysis can be highly sensitive to misspecification of the linkage model, several non-parametric analyses were performed.

2. Nonparametric linkage analysis

Affected pedigree member (APM) analysis was performed by the APM program, version 2.10 (Weeks and Lange 1988). This program uses marker information from affected individuals and tests whether affected individuals are more similar to each other at marker locus than would be expected by chance. The marker similarity is measured in terms of identity-by-state.

Non-parametric linkage analysis of the GENEHUNTER computer package was also used (Kruglyak et al. 1996). This analysis is model independent and effectively measure the extent to which marker alleles shared by descent between affected individual is greater than expected under random segregation. When an interesting result was found through two-point analysis, multipoint linkage analysis is often employed to maximize linkage information and localize the disease gene more precisely on an established map of markers. Multipoint linkage analysis with GENEHUNTER was performed in first three papers.

3. Transmission disequilibrium test

Transmission disequilibrium test (TDT), a family-based association analysis, was calculated by the software package ETDT version 1.4. TDT starts with couples who have one or more affected offspring. It is irrelevant whether either parent is affected or not. This test simply compares the number of transmitted parental allele (T) to affected offspring and non-transmitted parental allele (N). The result is unaffected by population stratification.

4. Association

Association analyses of genotypes, phenotypes (carriership) or at times allele frequencies were performed by simple X² analysis or Fisher exact test in case of small numbers.
5. Kruskal-Wallis ANOVA test and Mann-Whitney U-test

mRNA expression levels in two groups were compared by Mann Whitney U test and in more than two groups by nonparametric Kruskal-Wallis ANOVA.
SUMMARY OF THE INDIVIDUAL PAPERS

Analysis of peaks suggested by previous genomic screens (paper I, II)

Four published genome-wide screens in MS identified a number of candidate regions for susceptibility genes in addition to the HLA complex in 6p21. However, none of these regions provided formally significant evidence for genome-wide linkage, they need further supports. We investigated 12 such regions in 46 Swedish multiplex MS families, 28 singleton families, 190 sporadic MS patients and 148 normal controls by linkage and association analysis. One microsatellite marker, in 12q23, provided evidence for association besides suggestive transmission distortion and slightly positive linkage. In addition, a marker in 7ptr-15 showed a significant transmission distortion as well as a highly significant score in affected pedigree member analysis, but not quite significant deviation in association analysis. One of three markers in 5p, a region implicated in all four previous studies, showed a weakly positive lod score, but no other evidence of importance. Markers in rest chromosomal regions provided little or no importance for MS (see table 5). In summary, these data support the importance of genome-wide screens in the identification of new candidate loci in polygenic disorders.

Another genomic region 3p14-13 was identified as promising by the British and Canadian screens. This region contains the SCA7 gene, which may cause spinocerebellar ataxia, a neurodegenerative disease sharing some clinical features with primary progressive MS. Here, we used eight microsatellite markers covering 36 cM to search for linkage and association in 146 Nordic MS multiplex families and 190 Swedish sporadic MS patients. We obtained an NPL score of 2.39 for marker D3S1285, the highest so far in the Nordic (including Swedish, Danish, Norwegian and Finish) MS affected sib-pair families (see table 5).

Under the assumption that genetic heterogeneity may exist between different ethnic groups, we stratified the families according to ethnic origin, i.e. analyzed the two largest groups, the Danes and the Swedes separately. Among the 61 Swedish families, a slightly positive score (NPL=1.2) was observed for the D3S1573 marker, 20 cM telomeric to D3S1285 whereas the 59 Danish families revealed no positive score for this marker. Contrary, whereas the two-point NPL-score for the Danish families was positive (NPL=1.32) for the D3S1285 marker, the multipoint plot showed neutral scores that gradually increases going further centromeric. This may suggest that heterogeneity exist even within the Scandinavian population. Association analysis of these markers in Swedish MS patients revealed modest allelic associations of uncertain significance not supported by transmission analysis. A trinucleotide expansion analysis of the SCA7 gene failed to reveal expansions. We conclude that support was obtained
for the location of a gene or genes with importance for MS susceptibility in the 3p14-13 region.

**Analysis syntenic regions identified in autoimmune animal models (paper III)**

Genomic regions influencing disease have been mapped in various experimental organ specific inflammatory disease models. These susceptibility loci appear to overlap with each other suggesting that common genes or mechanisms are influencing different organ specific inflammatory diseases (Becker et al. 1998). We therefore hypothesized that analysis of syntenic regions in humans from experimental model QTL might lead to the identification of human susceptibility genes. We investigated eight chromosomal intervals syntenic to loci of importance for experimental autoimmune model diseases in rats in 74 Swedish MS families. Possible linkage (a highest NPL score of 1.16) was observed with markers in 12p13.3, a region syntenic to the rat Oia2 locus which is importance for OIA. Four markers in the T cell receptor β chain gene region in 7q35 showed possible linkage (highest NPL score also here 1.16). This locus is syntenic to the rat Cia3 locus. Both these two loci overlap with chromosomal regions showing indicative evidence for linkage in previously published MS genomic screens. Indeed, the Oia2 and Cia3 rat loci were recently found to be linked also with EAE, a commonly used model for MS. We conclude that the evidence for 12p13 and 7q35 to harbor genes of importance for MS is mounting. The synteny with experimental loci may eventually facilitate their identification.

**Candidate gene studies of CD40LG and IFNG (paper IV, V)**

The CD40-CD40 ligand receptor-ligand pair is involved in several immune events, in the regulation of both humoral and cell-mediated immune functions. Since our group and others have recently shown CD40 ligand to be highly expressed on the peripheral blood mononuclear cells (PBMC) of multiple sclerosis (MS) patients, and since activated helper T cells expressing CD40 ligand have been found in the brain sections of MS patients, the protein is believed to be involved in MS development and is an obvious candidate gene in MS. We studied the influence of a polymorphic dinucleotide-repeat marker located in the 3' untranslated region of the X-linked gene encoding CD40 ligand (CD40LG) on susceptibility to and disease severity in MS. From a total cohort of 771 Nordic definite-MS patients, the cohort’s most (n=92) and least disabled octiles (n=90), as well as random samples of intermediately disabled males (n=119) and females (n=121), were genotyped; 135 ethnically matched healthy subjects were used as controls. In addition, the effect of the polymorphism on CD40 ligand mRNA expression was assessed using PBMC from 54 MS patients and 22 controls. Phenotype frequencies for the CD40LG marker did not differ significantly between
gender-conditioned intermediate-MS subgroups and controls, or between gender-conditioned disability octiles. Nor did the polymorphism appear to exert any significant effect on mRNA expression in either patients or controls.

IFN-\(\gamma\) is a proinflammatory cytokine shown to have an important influence in MS pathogenesis. Previous analysis of a dinucleotide repeat in the first intron of this gene showed a surprising association with RA, (Khani-Hanjani et al. 2000) and one of its alleles, CA12, showed correlation with high IFN-\(\gamma\) protein production in vitro.(Pravica et al. 2000). Finally, in previous study by our group of 34 Swedish MS families (He et al. 1998) showed a promising two-point linkage analysis and a subsequent analysis of Swedish and Italian MS patients indicated a possible association with this marker. (Goris et al. 1999; Vandenbroeck et al. 1998) In light of these findings, we considered it relevant to reassess the possible importance of this multiallelic dinucleotide repeat in IFNG in larger numbers of MS families and patients.

We performed linkage and familial association analyses in 100 Nordic sibling pairs and a case-control association analysis on 220 intermediately disabled sporadic MS patients and 266 controls. To determine the effect of the polymorphism on disease outcome, we compared genotype frequencies in the most and least disabled octiles of a total cohort of 913 MS cases. We also measured IFN-\(\gamma\) mRNA levels in unstimulated peripheral blood mononuclear cells from 46 MS patients and 27 controls grouped according to IFNG intron 1 genotype. Both nonparametric linkage analysis and transmission disequilibrium testing of the 100 sibling pairs produced negative results. Genotype frequencies for intermediate-MS patients did not differ significantly from those for controls; nor did genotype frequencies in the benign-MS octile differ significantly from those in the severe-MS octile. Comparison of IFN-\(\gamma\) mRNA levels in genotype-conditioned subgroups revealed no significant differences (See figure 2). Thus, alleles at the IFNG intron 1 dinucleotide repeat appear to affect neither MS susceptibility and severity nor IFN-\(\gamma\) mRNA expression in PBMC.
Table 5. Overall linkage, association and expression results from 53 markers studied in the thesis

<table>
<thead>
<tr>
<th>Human chromosomal region</th>
<th>Marker name</th>
<th>Studied in paper (I-V, GSP, SR or CG*)</th>
<th>APM P value</th>
<th>NPL score</th>
<th>NPL P value</th>
<th>TDT P value</th>
<th>Case control P value</th>
<th>mRNA expression &amp; polymorphism</th>
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* GRP means genomic screen peaks, SR means syntenic region from animal model diseases, CG means candidate genes. N# indicates more than one comparison in the study, but all get negative finding.

**Figure.** IFN-gamma expression in MS patients (n=46) and controls (n=27)

![IFN-gamma expression in MS patients and controls](image)

Figure 2 IFNG genotype based mRNA expression in MS and controls
GENERAL DISCUSSIONS

Factors affecting genetic mapping of MS

The failure of characterizing MS genetically in spite of major efforts indicates the complexity of mapping genes in complex disease. The reasons for the complexity could be locus heterogeneity, allele heterogeneity, multiple gene involvement, phenocopies, reduced penetrance, late age onset, variable expression, anticipation, new mutations, gene-gene and gene-environment interactions. These characteristics of the disease may result in a situation where minor effect genes never become detected. Other factors may come from limitations in study design, for example, simply applied traditional linkage and association analyses may not consider various confounding factors, ie, using wrong genetic model in linkage analysis or small sample size in association analysis, and will easily lead to false positive or false negative results. (Type I error and type II error)

For overcoming adjustable factors, many steps should be considered in mapping a gene. By introducing parameters such as age dependent liability classes for penetrance, heterogeneity α and assuming different kinds of genetic models in linkage data is one way. Another way is to critically define the phenotype according to disease subtype, clinical course or disease severity to lessen the genetic heterogeneity. Stratifying the families based on some à priori characteristic before the analysis of linkage data greatly facilitated establishing linkage in certain disorders. Two such examples are Alzheimer disease and familial breast cancer (Goate et al. 1991; Hall et al. 1990; Levy-Lahad et al. 1995; Rogaev et al. 1995; Sherrington et al. 1995). Through stratifying for age of disease onset, disease genes were identified, demonstrating existence of genetic heterogeneity.

Proper study design considering ethnic differences, sample size, stratification to lessen confounding factors could also increase the chance of finding genes. So with higher sample size, stratification of patient groups, considering every aspect involved in the above-mentioned major steps in gene mapping will facilitate gene identification.

Why study syntenic regions

Becker’s meta-analyses of 23 different genomic scans for autoimmune or immune-related disorders in humans and animal models indicated a co-localization of susceptibility genes of different autoimmune diseases in human and animals. This includes insulin dependent diabetes mellitus (IDDM), MS, OIA, CIA and EAE etc (Becker et al. 1998). Evidence for common autoimmune disease genes controlling onset, severity and chronicity based on experimental models for MS and RA has been found
(Bergsteinsdottir et al. 2000). One interpretation of this kind of colocalization is that these loci harbor genes that are key regulators of pathogenic immune responses. Such genes would regulate autoimmune disease in a target organ independent fashion. These genes may be considered as “autogenes”. If autogenes exist, a familial aggregation of autoimmune disease in general could be expected. An epidemiology study shows there is tendency of this kind of clustering (Lin et al. 1998). We hope that studies of genes in animal models could help understanding disease pathways in human.

Mapping information in animal models can be refined by cross breeding to make congeneric strains, making it theoretically easier to identify susceptibility genes in animal models than in human disease. Transgenic techniques may then be used for functional characterization of newly identified genes, thereby uncovering aspects of disease pathogenesis. We are therefore especially encouraged by the apparent significance in MS of genomic regions defined in experimental inflammatory diseases where prospects for positional cloning of genes are promising. After exact positioning in rats or mice, human susceptibility genes may be readily identified. Our syntenic region study was based upon the above theory and our results support this notion, since 12p13-12 and 7q34-36 are among the loci pointed out by Becker (Becker et al. 1998).

Rationale of methods used in the present study

Strategies for complex disease mapping usually involves a combination of linkage and association techniques. In many ways linkage and association provide complementary data. Linkage operates over a long chromosomal range. However candidate regions defined by linkage are usually too large for positional cloning. Association tests like case-control analysis and TDT have the opposite characteristics. Computer simulations and empirical data have suggested that LD extends only a few kilobases (kb) around common SNPs, whereas other data have suggested that it can extend much further, in some cases greater than 100 kb (Kruglyak 1999; Reich et al. 2001). Therefore a genome screen by LD would involve huge numbers of tests; on the other hand, a positive result would locate the susceptibility factor rather accurately.

A natural study design is therefore to start with a genome-wide screen by linkage, probably in affected sib pairs, and then once an initial localization has been achieved, to narrow the candidate region by LD mapping.

In the first two papers, we were in the stage of retesting those genomic regions deserving more consideration among those revealed by genomic screens in MS, so, naturally, linkage analysis was in focus. In addition, we also performed association analysis for those markers, although we now consider it unlikely to detect the relevant association within such big regions by selecting only a few markers. So far, only linkage analysis has been completed in the syntenic region study (paper III). We intend to turn to association analysis once the animal loci have been better defined in congeneric strains.
For the last two papers, since we analyzed two well-studied intragenic markers, specific allelic associations were the main focus.

Power consideration

When reviewing genetic studies of MS and other complex disease, one often encounters the problem of lack of confirmation of previous reports. In general, findings in studies using epidemiological methodology frequently end up being impossible to confirm. This is often explained either by lack of statistic power (in the follow-up study) or methodological differences. However, the most frequent cause is that a reported observation was due to a type 1 error, i.e. a false positive finding.

We estimated that our Swedish family studies had the power of 66%, 85% and 95% power to detect linkage under an autosomal dominant model of 10%, 35% and 70% penetrance. But since in the real situation, many simulated conditions are not fulfilled, the chance of finding a true influence is still questionable. Risch (Risch and Merikangas 1996) estimated that the number of families needed for identification of a disease gene are beyond reach if the genotypic risk ratio is lower than 2.0. However, association analyses requires comparably smaller sample sizes. So further analysis in bigger sample sizes of our studied markers is suggested for our linkage analysis, for instance in meta-analysis of published data.

In testing an hypothesis, two types of error are encountered. Type I error (false positive: rejecting the null hypothesis when it is true) and type II error (false negative: accepted the null hypothesis when it is false). Thus, deficient sample size often leads to false negative result.

Adjustments for making multiple comparisons in large bodies of data are recommended to avoid rejecting the null hypothesis too readily. Unfortunately, reducing the type I error for null association increases the type II error for those associations that are not null. A large number of statistical comparisons have been made in our association analyses. The risk of not adjusting for multiple comparisons is to get false positive findings. Since most of our studies showed negative findings even before correction for multiple comparison, we think this is less of a problem. Actually, there are different opinions on whether a strict adjustment for multiple testing is necessary or not (Greenland and Robins 1991; Rothman 1990). Furthermore, scientists should not be so reluctant to explore leads that may turn out to be wrong that they penalize themselves by missing possibly important findings (Rothman 1990).

Limitation of the present study

Although our current study indicates both positive and negative finding in linkage and
association analysis, there are certain limitations of this study that hopefully will be solved in our future work.

Choosing one marker representing one chromosomal region or one marker in one gene may not be enough to exclude an importance of that region or specific gene. In linkage studies, maximum of peaks may not exactly represent the location of the disease gene which could be located anywhere in the region. According to the traditional rationale for an association analysis, deviations in genotype frequencies between cases and controls, or between patient subgroups, may indicate either that the investigated polymorphism itself plays a role functionally in the studied disorder, or that the polymorphism is in LD with one or more etiologically important polymorphisms nearby. The extent of LD, determined in part by the unique evolutionary history of the population sampled, has also been shown, in recent studies, to display, with populations, considerable variation across the genome—presumably, on account of natural selection. Thus, we cannot rule out the possibility that, by limiting our study to a single polymorphism in one gene, we were unable to detect a true association between the studied genes and MS susceptibility or severity. Thus to study all polymorphisms detected in a gene together using haplotype analysis would strengthen the conclusion.

Future directions

In the present studies, we identified six chromosomal regions in Swedish and Nordic families, worth further study. However, these findings were relatively weak. In comparison with the recently finished Nordic genomic screen, which was based on a larger sample size, partly overlapping with the families used in the present studies, findings did not correspond very well, i.e. positive peaks became weaker by additional families. Therefore, further analysis of these regions is still needed. Anyhow, since most of these loci have been supported by several studies, we anyway consider it relevant to turn to typing of SNP and microsatellite markers for LD and extended haplotype analysis near selected genes within these loci.

We know now that one reason for failure in linkage analysis in multifactorial disease is related to the disease itself being heterogeneous. This leads to a situation where we have difficulties in getting enough samples to carry out a study with sufficient power. Getting access to larger materials through even larger co-operations may be necessary. But inevitably this will further introduce heterogeneity of the studied group. The heterogeneity may exist in different ethnic groups, (our results in paper II support the assumption that there is heterogeneity between the Swedish and Danish groups) may exist in different MS families, so the chance of finding genes may be slim because of the characteristics of the disease itself. Pessimists even believe polygenic genes may never be found.

We admit to be a little disappointed by linkage results in MS so far, being not as
successful as we had hoped. But the efforts of finding genes is still carried on in the following ways: analysis of animal models; collection of further MS families and sporadic MS patients in the Swedish population; more trio-families; analysis of homogeneous samples in isolated population or even extended pedigrees like the one from “Överkalix”.

With the discovery of massive numbers of genetic markers in the past two years, the completion of the human DNA sequence and the development of better tools for genotyping, association studies seem come back to be the main approach in complex disease gene analysis. A European MS genetics consortium lead from Cambridge is carrying out a project in which thousands of MS patients are genotyped for 6000 micro-satellite markers across the genome in a pooled DNA manner. Our group is one of the participants. The hope is, that through this or other joint efforts, we will eventually see a break thought in MS genetics in the near future.
CONCLUSIONS

In this thesis work, after studying candidate genes and regions of interest in the Swedish and Nordic populations, we have got support for the importance of six chromosomal regions: 3p, 5p, 7p, 7q, 12p and 12q. These regions are some of a number of genomic regions, which may harbor MS susceptibility genes. These regions need further confirmation and delineation.

For the other chromosomal regions and genes studied, no evidence of linkage or association was found. But no evidence of linkage or association does not necessarily mean evidence of no linkage or association. Of course, our negative findings may have even less relevance for other populations, since heterogeneity is likely to exist between populations, different patterns of disease, or even between different time point of a disease.

Gene expression analysis of IFNG and CD40LG did not reveal that the studied polymorphisms had an importance for mRNA expression in PBMC. Thus we did not find support for a reported effect of the IFNG intron CA12 repeat on IFN-γ secretion. However, the studies differed in testing protein and mRNA respectively. Preferably, studies of both mRNA and protein in parallel should be chosen in the future.
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