Multiple Sclerosis: Studies of the Interferon system and search for infectious agents

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Multiple Sclerosis: Studies of the Interferon system and search for infectious agents

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Abstract

Multiple sclerosis (MS) is a chronic disease of the central nervous system, characterized by focal inflammatory lesions with demyelination and axonal loss. It is believed that MS is an immune-mediated disorder with both genetic and environmental contributions to the pathogenesis. Interferons (IFNs) are naturally occurring proteins known to have antiviral, antiproliferative and immunoregulatory effects. Treatment with IFN beta has a favourable effect in MS while IFN gamma was found to precipitate exacerbations. Previous studies reported MS-associated abnormalities both with regard to IFN production and responses to viral infections.We have investigated whether polymorphisms in the IFN system influenced susceptibility to MS, but found no linkage between any of the investigated loci and MS.

Approximately 70% of monozygotic twins are discordant for MS indicating that environmental factors contribute to the development of the disease. We investigated, in 3 pairs of monozygotic twins discordant for MS, serological differences that could be important for the development of MS. Two MS affected twins had serological evidence of a previous infection with Borrelia burgdorferi respective Toxoplasma gondii. In general the serum titers were strikingly similar in the twins, indicating no major disturbances of the humoral immune system in MS. Many types of viruses induce IFN production, but it is unclear whether IFN is produced in MS lesions. We searched for the expression of MxA, a cytoplasmic protein induced by IFNA/B, with the capability to inhibit the replication of several viruses. No anti-MxA staining was found in MS brain tissue indicating that MxA, and consequently IFNA/B, probably is not produced in these lesions.

Oligoclonal bands (OCBs) are detected in the CSF in the majority of patients with MS. They are believed to result from continuous immunization against unknown antigens. We investigated if antibodies in CSF from MS patients bound to structures in MS brain lesions and control brain tissue and found that brain lesions from secondary progressive MS patients contain a unique structure that binds IgG antibodies from the patients. Preincubation of brain sections with CSF from MS patients enhanced the IgG staining. Direct fluorescence labelled CSF antibodies from an MS patient showed similar binding pattern as with anti-IgG in MS lesions. No binding of fluorescence labelled antibodies was detected in control brain tissue. This indicates that the antigens/target structures are absent from normal brain. Double staining showed that the IgG was bound to the axons and was observed both in myelinated axons and in totally or partially demyelinated axons suggesting that the antibody binding may be an early event in myelin and axon destruction.

Key words: Multiple sclerosis, interferon, genetic linkage, twins, virus, MxA, cerebrospinal fluid, immunoglobulin, axon, demyelination

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To Pierre

Contents

Original articles

This thesis is based on the following papers, which are referred to in the text by their roman numerals (I – V).

I Bergkvist M, Martinsson T, Åman P and Sandberg-Wollheim M. No genetic linkage between multiple sclerosis and the interferon alpha/beta locus. J. Neuroimmunology 1996; 65:163-65.

II Bergkvist M, Olsson M and Sandberg-Wollheim M. No evidence for genetic linkage between development of multiple sclerosis and components of the IFN-system and the JAK-STAT pathway. Multiple Sclerosis 2004 Feb; 10 (1):87-8.

III Bergkvist M and Sandberg-Wollheim M. Serological differences in monozygotic twin pairs discordant for multiple sclerosis. Acta Neurol Scand 2001: 262-265.

IV Bergkvist M, Nordborg C and Åman P. Immunohistochemical analysis of MxA, p53 and CHOP expression in brain tissue from patients with multiple sclerosis. Manuscript.

V Bergkvist M, Nordborg C, Oldfors A and Åman P. Identification of target structures for autoantibodies in brain tissue from patients with multiple sclerosis. Manuscript.

Abbreviations:

APP	Amyloid precursor protein
CDMS	Clinically definite multiple sclerosis
CHOP	C/EBP-homologous protein
CMV	Cytomegalo virus
CNS	Central nervous system
CSF	Cerebospinal fluid
CT	Computerized tomography
DNA	Deoxyribonucleic acid
EAE	Experimental allergic encephalomyelitis
EBV	Epstein-Barr virus
NF	Neurofilament
HERV	Human endogenous retrovirus
HHV	Human herpes virus
HLA	Human leukocyte antigen
HSV	Herpes simplex virus
HTLV	Human T lymphotropic virus
IFN	Interferon
IFNA	Interferon alpha
IFNAR	Interferon alpha receptor
IFNB	Interferon beta
IFNG	Interferon gamma
IFNGR	Interferon gamma receptor
IgG	Immunoglobulin G
IP	Immunoprecipitation
IRF	Interferon regulatory factor
ISGF	Interferon stimulated gene factor
ISRE	Interferon stimulated response element
JAK	Janus family of tyrosine kinase
GAF	Gamma activated factor

GAS	Gamma activated site
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MSRV	Multiple sclerosis associated retrovirus
MS	Multiple sclerosis
NAA	N-acetyl aspartate
NK	Natural killer
OCBs	Oligoclonal bands
ON	Optic neuritis
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PML	Progressive multifocal leukoencephalopathy
PPMS	Primary progressive multiple sclerosis
RNA	Ribonucleic acid
RRMS	Relapsing remitting multiple sclerosis
RT-PCR	Reverse transcriptase polymerase chain reaction
SPMS	Secondary progressive multiple sclerosis
SSPE	Subacute sclerosing panencephalitis
STAT	Signal transducer and activator of transcription
TYK	Tyrosine kinase
VEP	Visual evoked potential
VZV	Varicella zoster virus

Summary

Multiple sclerosis (MS) is one of the most common neurological diseases affecting young adults. It is a chronic disease of the central nervous system, characterized by focal inflammatory lesions with demyelination and axonal loss. It is believed that MS is an immune-mediated disorder with both genetic and environmental contributions to the pathogenesis.

Interferons (IFNs) are a group of naturally occurring proteins known to have antiviral, antiproliferative and immunoregulatory effects. Treatment with IFN beta (IFNB) has a favourable effect on MS while IFN gamma (IFNG) was found to precipitate exacerbations. Previous studies reported MS-associated abnormalities both with regard to IFN production and responses to viral infections. In paper I and II we have investigated whether polymorphisms in the IFN system influenced susceptibility to MS, but found no linkage between any of the investigated loci and MS.

More than 70% of monozygotic twins are discordant for MS indicating that environmental factors contribute to the development of the disease. Since monozygotic twins are genetically identical, discordant twins are ideal for searching for triggering events. In paper III we investigated, in 3 pairs of monozygotic twins discordant for MS, serological and environmental differences that could be important for the development of MS. Two MS affected twins had serological evidence of a previous infection with *Borrelia burgdorferi* respective *Toxoplasma gondii*. In general the serum titers were strikingly similar in the MS-affected and nonaffected twins, indicating that MS is not associated with major disturbances of the humoral immune system.

Many types of viruses induce interferon production, but it is unclear whether interferon is produced in MS lesions. In paper IV we searched for the expression of MxA, a cellular endoplasmic reticulum protein specifically induced by IFNA/B, with the capability to inhibit the replication of several viruses. We found no anti-MxA cytoplasmic staining in brain tissue from secondary progressive MS patients and concluded that MxA, and consequently IFNA/B, probably is not produced in these

lesions. If local virus infections are involved, they seem to fail to induce any IFN response.

Oligoclonal bands (OCBs) are detected in the CSF in the majority of patients with MS. They are believed to result from continuous immunization against unknown antigens. In paper V we investigated if antibodies in CSF from MS patients bound to structures in MS brain lesions and control brain tissue from patients without neurological disease. We found that brain lesions from secondary progressive MS patients contain a unique structure that binds IgG antibodies from the patients. Preincubation of brain sections with CSF from MS patients enhanced the IgG staining of these structures. Direct fluorescence labelled human CSF antibodies from an MS patient showed similar binding pattern as with anti-IgG in MS lesions. No binding of fluorescence labelled antibodies was detected in control brain tissue. Altogether this indicates that the antigens/target structures are absent from normal brain. Double staining showed that the IgG was bound to the axons and was observed both in naked demyelinated axons and in axons fully or partially covered with myelin. This suggests that the antibody binding may be an early event in myelin and axon destruction.

Introduction

Multiple sclerosis (MS) is one of the most common neurological disabling diseases among adults under 40 years of age, affecting around 0.1% of northern Europeans.[1] It is a chronic inflammatory demyelinating disease of the central nervous system (CNS) where axonal loss is likely to contribute to much of the disability. The symptoms are diverse as any part of the CNS may be affected. Although the aetiology of MS is unknown, genetic factors, environmental agents and autoimmunity to myelin or axonal structures, may all contribute to the pathogenesis.

The increased recurrence rate in genetically related family members, the greater concordance among monozygotic than among dizygotic twins, and the occurrence of isolated ethnic groups with resistance to MS, point to inheritable determinants of susceptibility.[2-4] Key components of the immune system have been proposed as potential susceptibility loci, but the only consistent finding from whole genome linkage screenings has been the association with alleles of the human leucocyte antigen (HLA) system on chromosome 6p21.[5, 6]

Geographic distribution, data from migration studies and the fact that most monozygotic twins are discordant for MS, indicate that environmental factors contribute to the development of the disease. MS may be related to infectious agents that act either directly or through autoimmune mechanisms in genetically susceptible populations.[7, 8]

The characteristics of MS lesions are suggestive of slow or latent virus infection. Herpesvirus and retrovirus have been suggested as potential candidates in the pathogenesis of MS since both are neurotropic, establish long-life latent infections and cause primary demyelination.[9, 10] However, no single unique virus to date has definitively been associated with this disease.[11, 12]

Several observations suggest that the interferon system may be of special interest in MS development. The Interferon (IFN) family comprises type I (alpha and beta, IFNA/B) and type II (gamma, IFNG). Type I and II IFNs bind to different receptors but both activate JAK-STAT signal transduction pathways that control

the transcription of IFN inducible genes.[13] Treatment with Interferon beta (IFNB) in relapsing remitting MS (RRMS) has been shown to reduce exacerbations and accumulation of magnetic resonance imaging (MRI) abnormalities in a majority of patients.[14-16] Treatment with interferon gamma (IFNG), on the other hand, precipitates exacerbations.[17] The mechanism behind the IFN effects in MS remains partly unclear but both immunomodulatory [18] and antiviral effects of IFN are believed to have a therapeutic potential in MS treatment.

Many types of virus infections are known to induce IFN production.[19] IFNA/B is produced in direct response to virus infections. IFNG, on the other hand, is synthesized in response to the recognition of infected cells by activated T lymphocytes and natural killer (NK) cells rather than directly by virus infection. [19]

Previous studies have reported abnormal immune responses to a variety of viruses in MS patients [20, 21] but also abnormalities in the IFN responses.[22-25]

Persistent intrathecal immunoglobulin (Ig) production is a standard feature in the majority of MS patients and is characterized by elevated IgG index and presence of oligoclonal bands (OCBs) in the cerebrospinal fluid (CSF).[26, 27] This is believed to be the result of a repeated immunization against one or a limited number of unknown antigens. Oligoclonal IgG bands are present at an early stage of the disease. They differ from patient to patient but the pattern remains fairly constant in a given patient during the course of the disease.[27] This suggests that there is a continuous activation and recruitment of B lymphocytes to the CNS and that antigens and immune complexes could be present in the brain tissue of MS patients.

This thesis was undertaken to investigate whether polymorphisms in the genes of the IFN system influenced susceptibility to MS and to search by serological, molecular biological and immunohistochemical methods for infectious agents and to identify target structures for autoantibodies in brain tissue from patients with MS.

Background

Clinical and diagnostic aspects

Multiple sclerosis is an important cause of progressive neurological disability. In most Caucasian populations MS is second only to trauma as a cause of acquired neurological disability in young adults between 15 and 45 years. MS occurs two to three times more often in women than in men and affects more than 2.5 million people world-wide.[28]

MS is characterized by myelin loss, gliosis and varying degrees of axonal pathology. Myelin destruction impairs the saltatory conduction along axons that is necessary for normal functioning of nerve impulses. Patchy degenerative inflammatory changes occur within the brain and spinal cord resulting in diverse symptoms as any part of the CNS may be affected. Common symptoms include visual disturbances, loss of balance and coordination, sensory and motor disturbances, bladder and bowel incontinence, pain and fatigue. Over 50% of MS patients develop cognitive impairment which may indicate also a cortical involvement of the lesions.[29-31]

The clinical course is unpredictable and variable. MS is classified as relapsing-remitting (RR), secondary progressive (SP) or primary progressive (PP) MS. Approximately 85% start with a relapsing-remitting course where complete recovery usually follows the initial relapses but after later exacerbations an increasing disability often persists and in the majority of patients secondary progression subsequently develops. A small group among those with relapsing remitting MS have a benign course. About 15% are primary progressive from onset.

In recent years it has been demonstrated that functional impairment correlates better with axonal than myelin injury. N-acetyl aspertate (NAA) is a marker of axon damage in the white matter, identified as a specific peak by magnetic resonance spectroscopy (MRS). It has been observed that low NAA levels in an acute lesion often normalize during recovery, demonstrating that axonal dysfunction is not always irreversible.[32] However, although axonal loss may have no clinical consequences for many years, irreversible neurological disability develops when a threshold of axonal loss is reached and compensatory CNS resources are exhausted.[30, 33]

The diagnosis of MS rests on the objective demonstration of dissemination of lesions in both time and space. Magnetic resonance imaging (MRI) is integrated with clinical and other diagnostic methods such as CSF analysis and sometimes visual evoked potentials (VEP).

Genetic susceptibility in multiple sclerosis

In MS, susceptibility to the disease is believed to be inherited. The risk in first degree relatives (siblings and children) to develop MS is 3-5 %, which is 30-50 times the 0.1% risk for the general population.[34] A higher concordance rate for MS among monozygotic twins, (25-27 %) compared with dizygotic twins, (3,5-5,4 %) [35-37] indicates genetic components in the aetiology of MS.

Studies of half siblings [3, 4] and adoptees [38] also point to inheritable determinants of susceptibility. The prevalence of MS among spouses and adopted children of MS index cases is similar to that in the general population. Dyment et al. conclude that genetic sharing and not family environment is critical for the familial aggregation of MS.[4]

There are ethnic groups that remain relatively or entirely free from MS despite living in a high prevalence area. Examples of this are, Samis in the northernmost Norwegian populations,[39] Maoris in New Zeeland [40] Gypsies in the county of Baranya in Hungary and North American Indians living in Canada.[41]

Many candidate genes in the immune system such as those regulating cytokines, immune receptors and myelin components have been suggested to be important for susceptibility to MS. The only locus showing a strong and consistent evidence for this is within the major histocompatibility complex (MHC) on chromosome 6p21.3. Associations with the HLA-DR2 haplotype (DRB1*1501-DRB5*0101-DQA1*0102-DQB1*0602) have been demonstrated in several populations, particularly those of Northen European decent.[42, 43] However much of the genetic effect in MS remains to be explained as the susceptibility attributed to the HLA locus in MS is estimated to between 17 % and 62%.[44] Several whole genome linkage studies have been performed [45-51] but no single major genetic element has been found to confer disease susceptibility.

Environmental aspects

The frequency of MS is higher in temperate zones and in Western European populations (around 100 cases/100000) and lower in tropical and subtropical areas (<5 cases/100000 in Africa and Asia).[41] Epidemiological studies show an uneven geographical distribution of the disease with a north–south gradient in Europe, Canada and Northern USA and a similar but less pronounced gradient in the opposite direction in the southern hemisphere.

Migration studies suggest that the age at the time of exposure to environmental factors appears to influence the risk of an individual developing MS.[52]

Observations from epidemics of MS in Iceland and the Faroe islands suggest that MS may be a transmissible infectious disease.[53, 54]

Recently, Willer et al reported an association between month of birth and risk of MS, implying interactions between environment related to climate and genes.[55]

Because the prevalence of MS is high in areas where environmental supplies of vitamin D are low, the role of vitamin D has also been discussed. Ultraviolet sunlight is too low to produce adequate amounts of vitamin D_3 at high latitudes during the winter and a protective effect of vitamin D on risk of developing MS has been suggested.[56] In line with this, administration of the active metabolite 1,25-$(OH)_2$D has been shown to reduce disease activity in mice with experimental allergic encephalomyelitis (EAE), an animal model of MS.[57]

Finally, the fact that more than 80% of MS patients have no affected relatives and that most monozygotic twins are discordant for MS, underlines the importance of noninheritable factors.[58, 59]

Multiple sclerosis and virus

Several viruses are able to induce persistent demyelinating disease within the CNS. In humans the most prominent are progressive multifocal leukoencephalopathy (PML), caused by JC virus destruction of oligodendrocytes, and subacute sclerosing panencephalitis (SSPE), a disease in children and young adults as a rare complication of measles virus infection.[60] In MS the aetiology remains unknown. A viral involvement is possible because most human and animal demyelinating diseases of known aetiology are viral.[60] The human herpes viruses (HHV), (Herpes simplex virus type 1 and 2 [HSV 1&2],

Varicella zoster virus [VZV], Human herpes virus 6 [HHV-6], Epstein-Barr virus [EBV] and Cytomegalovirus [CMV]) are of special interest in MS. After primary infection with these viruses during childhood or adolescence, a lifelong infection may follow a latent-recurrent mode similar to the relapsing-remitting course of MS. Most herpesviruses cause persistent infections in the CNS, some are axonally transported in the neurons and several can induce demyelinating disease in the CNS.[10] Treatment with the antiherpes drug Acyclovir had no effect on neurological function of patients with MS in a placebo-controlled trial.[61] The exacerbation rate was reduced by 34% compared with placebo but statistical significance was not achieved. However, when stratified according to prestudy exacerbation rate, there was a significant treatment benefit in the group with high prestudy exacerbation rates. Anti-herpes virus therapy with Valacyclovir given to patients with relapsing–remitting MS reduced disease progression in patients with high levels of MRI-evident disease activity, but in most patients with MS this effect was not seen.[62]

HHV-6 has recently been associated with MS. Several studies compared the presence of HHV-6 in brain tissue, CSF and peripheral blood mononuclear cells in patients with and without MS. The results are conflicting, however. In all studies of HHV-6 by PCR, the virus was present in brain tissue from both MS patients and controls.[9, 63] Similar contradictory findings are reported with HHV-6 immunohistochemistry in the brain, DNA analyses in serum by PCR, HHV-6 antibody and HHV-6 lymphoproliferative responses. Clark *et al*. conclude that the lack of consistent findings suggest that HHV-6 infection alone is not sufficient for the development of MS.[64]

Epstein-Barr virus (EBV), the cause of infectious mononucleosis, has also been implicated in MS. The virus is known to be latent in B cells and seroconversion occurs before or during puberty into adult life, thereby matching epidemiological evidence with the time of exposure to disease triggering agents of MS.[65] All patients with MS have antibodies against EBV compared with 86-95% of controls. It is not known whether infection with EBV is a prerequisite for the development of MS or if 100% seropositivity is a consequence of MS. Wandinger *et al*. suggests that EBV might play an indirect role in MS as an activator of the underlying disease process.[66] However, no studies have attempted to show that the oligoclonal IgG in MS brain and CSF is directed against EBV. Hilton *et al.*

found no EBV-specific RNA in the brains of ten patients with MS, investigated with *in situ* hybridisation.[67]

The family of retroviruses including Human T lymphotropic virus (HTLV1) has for a long time been implicated in the pathogenesis of MS, in part due to the clinical and pathological similarities between HTLV1 associated myelopathy / tropical spastic paraparesis (HAM/TSP) and progressive MS.[68] Serologic and polymerase chain reaction (PCR) based findings suggested an association between HTLV-1 and MS.[69, 70] However, large-scale control experiments did not support this hypothesis.[71, 72]

Human endogenous retroviruses (HERVs) have also been implicated in the aetiology of MS.[73] HERVs comprise up to 1% of human DNA and have been suggested as triggers in a variety of autoimmune disorders where autoantibodies are suggested to crossreact with HERV proteins.

MSRV (multiple sclerosis associated retrovirus) is a retrovirus derived from EBV-infected B cells as well as from choroid plexus cells of MS patients.[74] MRSV sequences have been found in serum from both MS patients and healthy donors but were more frequently expressed in the former.[75]

In addition to herpes and retrovirus, several common childhood infections have been proposed to be involved in MS and no one virus has received more consideration than measles virus. However, measles virus RNA has not been detected in MS lesions even by sensitive reverse transcriptase polymerase chain reaction (RT-PCR) methodologies. Still some researchers suggest that because of a potential for molecular mimicry, virus may initiate autoimmune disease that can continue in the absence of the original pathogen.[76]

To date no single virus has been shown to be definitively associated with MS. It is possible that multiple viruses are involved in the aetiology of MS or that different viruses trigger the disease in different subsets of individuals.

Multiple sclerosis and interferon

Interferons (IFNs) are a group of naturally occurring proteins known to have antiviral, antiproliferative and immunoregulatory effects.[77] IFNs appear in the blood in response to infection or in response to antigen and mitogen stimulation but can not be measured in the circulation of healthy individuals.

The Interferon family comprises type I and II IFNs. Type I, also known as viral IFN, is induced by viral infections. In man it includes IFN alpha (IFNA) produced by leukocytes, IFN beta (IFNB) produced by most cell types but particularly by fibroblasts, and IFN omega. IFN omega is closely related to IFNA with which it shares 60 % protein sequence identity. Most types of virus infected cells can synthesize IFNA/B in cell culture.

Type II, also known as immune IFN, is induced by mitogenic or antigenic stimuli and consists of IFN gamma (IFNG). IFNG is synthesized by specific cells of the immune system such as natural killer (NK) cells and T lymphocytes.[78]

In man there are >15 IFNA genes, 1 IFNB and 1 IFN omega gene.[79] All lack introns and are clustered on the short arm of chromosome 9. The IFNG gene is located on the long arm of chromosome 12 and has three introns.[77]

The IFNs act by binding to specific cell surface receptors. IFNA and IFNB share and compete for the same receptor complex. The IFNA/B receptor (IFNAR) gene is located on chromosome 21q22.1 (subunit IFNAR1 and IFNAR2). The subunits of the IFNG receptor (IFNGR) gene are located on 6q23-24 (IFNGR1) and 21q22.1 (IFNGR2), closely linked to the IFNAR1 locus.

Although type I and II IFNs bind to different receptors, both activate the JAK-STAT signal transduction pathways, which control the transcription of IFN inducible genes. The tyrosin kinase JAK 1 interacts with the IFNAR-2 subunit of the IFNA/B receptor as well as with the IFNGR-1 subunit of the IFNG receptor.[77]

Binding of IFNA or IFNB to its receptor results in the activation of the receptor-associated tyrosine kinases Jak1 and Tyk 2, which is followed by tyrosine phosphorylation of STAT proteins. The activated STAT proteins dimerizate and together with IFN regulatory factor 9 (IRF-9), also known as p48, form the trimeric IFN stimulated gene factor 3 (ISGF-3) complex. The complex of these three proteins translocates to the nucleus and binds to the IFN-stimulated response elements (ISREs) present in most genes responsive to IFNA and IFNB. This results in the enhancement or inhibition of the expression of many cellular genes.[80]

In a similar way, binding of IFNG to its receptor results in the activation of the receptor-associated kinases JAK1 and JAK2, followed by tyrosin fosforylation of STAT proteins. The activated STAT proteins form homodimers, also called gamma-activated factor

(GAF), translocate to the nucleus, bind to specific elements, gamma-activated sites (GAS) of IFNG inducible genes, and stimulate transcription.

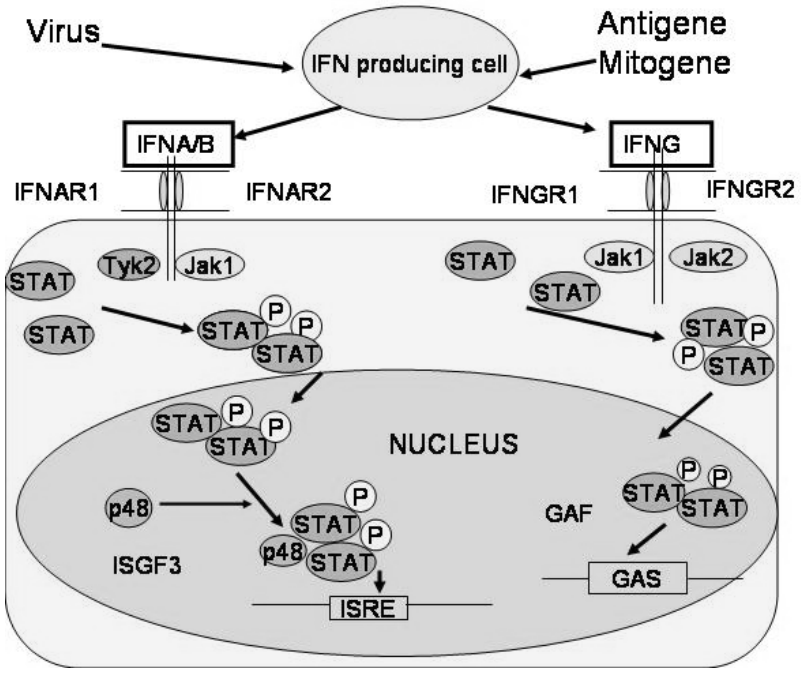

Figure 1. IFN production and action

Several of the genes of the IFN and JAK-STAT pathway systems exhibit genetic polymorphism, which may result in functional differences.[81-83]

Herzog reported abnormal IFN responses in MS patients. During an 18-month period intermittent interferonemia was detected in 88% of MS patients but in none of the controls.[23] Other studies have failed to detect IFN in CSF or serum in MS patients.[84] Neighbour reported significantly lower IFN response *in vitro* to measles virus and to other inducers of both IFNA/B and IFNG in cells from MS patients compared to the response in cells from healthy controls.[21]

Feng *et al*. found abnormal IFN signalling with low levels of IFN regulatory factor 1 and 2 (IRF1 and 2) which control multiple IFN-stimulated genes.[85]

Interferon produced by virus infected cells binds to neighbouring cells and triggers the expression of a number of genes that render the cells resistant to many types of viruses.[16] In this way the interferon response represents an early host defense, one that occurs prior to the onset of the immune response.

Also infection with bacteria, mycoplasma and protozoa induces IFN gene expression. Certain cytokines and growth factors may also induce IFN production.[80]

In addition to antiviral activity, the IFNs affect other processes such as those regulating cell growth, differentiation, apoptosis and modulation of the immune response. It is still not clear, however, whether interferon is produced in MS lesions and attempts to stain for interferon producing cells have been difficult, probably because interferon is rapidly secreted and not accumulated within the producing cell. MxA, the product of the *MX1* gene, is an interferon induced cellular endoplasmic reticulum protein with a molecular weight of about 75kD. It has an intrinsic GTPase activity and blocks viral replication.[86] MxA protein is not present constitutively in normal cells but is specifically induced by class I IFNs. It is not induced by the inflammation associated IFNG. MxA inhibits the replication of several viruses.[87] Quantitative MxA protein bioassays have been used to monitor the effects of IFNB treatment in MS patients.[88-90]

Although initially designed as an antiviral drug, the therapeutic benefit of IFNB in the treatment of MS appears to be based on immunomodulatory mechanisms. IFNB alters the balance of immunoregulatory and pro-inflammatory cytokines, inhibits Th1 and stimulates Th2 cytokines. IFNB down regulates T cell resistance to apoptosis and inhibits production of matrix proteinase 9.[91-95] In addition, IFNB has been shown to down regulate the expression of adhesion molecules on the cell surface.[18, 96]

Treatment with IFNG has been shown to precipitate exacerbations in relapsing remitting MS patients.[17] There was a significant increase in circulating monocytes expressing class II (HLA-DR) surface antigens, and in nature killer cell activity, suggesting that the attacks induced by IFNG treatment were immunologically mediated. IFNB is thought to counteract many of the actions of IFNG.[97-101]

Multiple sclerosis and stress induced proteins

Some MS lesions are histopathologically characterized by oligodendrocyte loss that in part could be caused by apoptosis.[104] On the other hand, apoptosis may be important in the elimination of self-reactive T cells.[105] To investigate genetic differences in apoptosis regulating factors, Kuhlmann *et al*. analyzed polymorphisms in the *TP53* gene but found no difference between RRMS and controls.[106]

The tumor suppressor gene *TP53* encodes a transcription factor, p53, with the ability to induce cellcycle arrest and apoptosis in response to different stress stimuli. Activation of p53 is induced by DNA damage and chemotherapeutic drugs, but also by inflammatory cytokines and viral infections, both postulated to be involved in the MS disease process. [102, 103] In most normal cells p53 is present at low levels in a dormant form, and requires activation to become fully functional.

Takaoka *et al*. showed that in mouse, transcription of the *TP53* gene is induced by IFNA/B based on the observation that IFNB increased the levels of p53 in cultured mouse cells. IFNG failed to activate the *TP53* transcription. Furthermore Takaoka showed that p53 in mouse, is activated in cells infected by vesicular stomatitis virus, Newcastle disease virus and herpes simplex virus, suggesting that activation and induction of apoptosis by virus infection represents an antiviral defense mechanism.[107, 108]

Immunostaining for the p53 protein in brain autopsy and biopsy material including MS tissue has yielded conflicting results. Yaziji *et al*. found no p53 immuno-reactivity in lesions of demyelinating disease.[109] Kurtkaya-Yapicier on the other hand, reported weak p53 expression by immunohistochemistry in astrocytes and oligodendroglia in biopsies from demyelinating diseases.[110] Wosik reported that active MS lesions (both biopsies and autopsies) revealed increased p53 expression in oligodendrocytes in lesions that featured oligodendrocyte apoptosis and cell loss, but not in lesions displaying oligodendrocyte preservation.[102]

CHOP also known as *GADD 153* or *DDIT3* is another stressinduced gene not previously investigated in MS. *CHOP* encodes a transcription factor that is induced in different stress conditions such as DNA damage, endoplasmatic stress and starvation.[111] The CHOP

protein is expressed at low levels in normal brain tissue, but strong nuclear expression is induced in cells only after DNA damage or other types of cellular stress.[112]

Neuropathology

Neuropathological analysis from a large number of samples of active MS lesions have suggested four different patterns of demyelination.[104, 113] Beside a T cell and macrophage dominated inflammatory immune response in all lesions, differences were found regarding immunoglobulin and complement deposition, myelin loss and oligodendrocyte degeneration. The patterns of demyelination were heterogeneous between patients, but were homogenous within multiple active lesions from the same patient.[104]

Recent studies have shown the presence of axonal injury both in acute lesions with inflammation and demyelination and in chronic lesions.[114, 115] Axonal damage seems to be an early phenomenon that begins already at disease onset, but the mechanisms of axonal injury and dysfunction are unknown. Trapp *et al*. showed that axonal transection occurred in active lesions and in patients with very short disease duration, suggesting the involvement of inflammation.[33, 116] In progressive disease, chronically demyelinated axons have been suggested to degenerate due to lack of myelin-derived trophic support.[30] Evidence of axonal injury in MS has been shown by different methods. Immunostaining using antibodies against markers for injured axons such as nonphosphorylated neurofilament (NF) [116] and amyloid precursor protein (APP) [114] supports axonal destruction. Magnetic resonance spectroscopy (MRS) provides information on axonal pathology by analyzing the chemical composition in brain tissue.[117] The reduction of the ratio N-acetyl aspartat (NAA) to creatine (Cr) (NAA/Cr) in the white matter in MS has been attributed to axonal loss / degeneration. CT and MRI show atrophy of the brain and spinal cord in MS patients.[118] Although there is evidence of axonal injury in MS, it has usually been regarded as an event secondary to myelin damage. However in recent years the hypothesis has been raised, that axonal pathology may be primary, or independent of, rather than secondary to demyelination and inflammation.[32, 119, 120]

Tsunoda and Fujinami propose an “inside-out” model for the pathogenesis of demyelinating diseases, suggesting a primary axonal injury, perhaps virus induced, that

triggers demyelination and leads to immunopathology. The injured axon is not regarded soley as the end result of pathology; rather they suggest that axonal injury could contribute to the spread of secondary damage, including demyelination.[121]

Oligoclonal bands and antibody responses in multiple sclerosis

The emphasis on T cells in the pathological process in MS has been derived mainly from the detection of activated T cells in MS plaques and the fact that the animal model of MS, experimental allergic encephalomyelitis (EAE) can be passively transferred by myelin reactive T cells. [122]

In recent years the role of B cells, plasma cells and immunoglobulins in MS has been reexamined. IgG producing plasma cells are found frequently in brain tissue from MS patients.[123] Different explanations of the B cell activation in MS have been suggested. It could be the result of antigenic stimulation targeted at specific molecules. Antibody production may also be driven by cross reactivity with infectious agents (molecular mimicry). Alternatively, the activation of B cells could be a random bystander effect of the inflammatory response in the lesions.[27]

Persistent intrathecal immunoglobulin (Ig) production is a standard feature in the majority of MS patients and is characterized by elevated IgG index and the presence of oligoclonal bands in the CSF.[124] Prospective investigations have shown that the presence of three or more clinically silent MS-like brain MRI lesions together with the presence of OCB´s in CSF in patients with monosymtomatic optic neuritis are strongly predictive of the future development of MS.[125] About 5 % of Caucacian MS patients have no OCB´s. These patients are believed by some to have a better prognosis according to a low plaque burden, [27] while others finds the quantification of oligoclonal bands to be an insensitive prognostic marker.[126] OCB patterns differ from patient to patient but remain fairly constant in a given patient during the course of the disease, although CSF IgG, expressed as percentage of total protein, fluctuates in individual MS patients.[127] Administration of corticosteroids reduces the elevated intrathecal IgG synthesis rate towards normal values but the oligoclonal IgG band pattern remains.[128] Based on oligoclonal banding patterns of IgG eluted from individual plaques, some investigations demonstrated different patterns in different plaques within the same MS brain, suggesting that the IgG is the result of random

B cell activation.[129] In contrast, other studies showed that each MS brain displayed the same pattern in all plaques, suggesting that the IgG in MS plaques are antibodies resulting from antigen driven stimulation.[127] Sandberg-Wollheim *et al*. followed the intrathecal immune response for 2,5-12 years in 10 patients from the early stage of MS. In 6 patients the changes in the patterns of intrathecally synthesized viral antibodies were characterized by the appearance of some "new" antibody populations and the waxing and waning of others, indicating transient as well as permanent recruitment of B cell clones producing viral antibodies of different specificities.[130]

In many CNS infections of known aetiology, most OCB´s have been shown to contain antibodies against the aetiologic agent, e.g. in SSPE, rubella panencephalitis, mumps meningitis, neurosyphilis, tuberculosis meningitis. In MS intrathecal antibody synthesis has been suggested against various viruses such as mumps, rubella, measles, varicella zoster and herpes simplex.[131] Still, the origin of these antibodies is unclear. There is no strong evidence of a relationship to the aetiology of MS, since de novo replication of the corresponding viral genome has not been found [132] and it has not been possible to trace the intrathecally synthesized viral antibodies to the OCBs present in CSF [133]

Autoantibodies recognizing several myelin proteins including myelin basic protein (MBP), proteolipid protein (PLP), myelin associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG), 2´3'-Cyclic nucleotide 3'phosphodiesterase (CNP) and oligodendrocyte-specific protein (OSP) as well as antibodies against glycolipids such as galactocerebroside are found in MS patients, but antibody reactivity against these antigens does not correspond to major electrophoretic bands. Proof that OCBs are directed against a particular antigen target requires the demonstration of their absorption by the putative antigen.[27]

In recent years the presence of autoantibodies directed against axonal components has been investigated and autoantibodies against e.g. the neurofilament light unit (NF-L) has been suggested.[134-136]

Aims

The aims of this thesis were:

To investigate the possibility of a genetic linkage between polymorphic loci in or close to the IFN alfa/beta, IFN gamma, IFN gamma receptor, IFN alpha/beta receptor, JAK1, STAT1 and STAT3 genes and MS in a familial material. (Paper I and II)

To study serological differences that could be relevant for the development of MS in 3 pairs of monozygotic twins discordant for MS. (Paper III)

To search for the expression of the interferon induced MxA protein and the two stress induced proteins p53 and CHOP in brain tissue from deceased patients with secondary progressive MS. (Paper IV)

To search for bound antibodies in MS lesions and to investigate if CSF derived antibodies from MS patients could bind to structures in MS and normal brain tissue. (Paper V)

Materials and methods

Paper I and II

We studied a large number of individuals belonging to unrelated families in whom at least two members had clinically definite MS (CDMS).

Healthy blood donors of the same ethnic origin but unrelated to the MS families were used as controls.

In paper I there were 126 individuals from 24 families. The patients were 18 men and 33 women. Unaffected family members were 41 men and 34 women. Controls consisted of 63 men and 78 women.

In paper II there were 140 individuals from 27 families. The patients were 20 men and 37 women. Unaffected family members were 45 men and 38 women. Controls consisted of 65 men and 83 women.

DNA was isolated from peripheral blood. Micro satellite polymorphic sequences in or close to four IFN gene loci and three JAK-STAT loci were amplified by PCR using fluorescence-tagged primers. The products were analysed on a capillary sequencer (Applied biotechnology type 377).

Paper III

Two male pairs and 1 female pair of twins with confirmed monozygosity, were investigated. The affected twins had clinically definite MS by standard criteria, supported by typical MRI lesions and oligoclonal bands in the spinal fluid. Medical and social histories were obtained by questionnaires. Serum samples were obtained and assayed for antibodies against 21 viruses, 4 bacteria and *Toxoplasma gondii* using immunoassays, neutralization tests, and EIA. The analyses were performed at the department of clinical microbiology, University Hospital, Lund.

Paper IV

Immunohistochemistry (IHC): Paraffin embedded brain tissue (autopsy material from six secondary progressive MS patients and one control patient without neurological disease) was stained with primary antibodies for MxA, p53, CHOP and CD3. Bound antibodies were visualized using the streptavidin biotin labelled (LSAB) peroxidase complex. Mayer was used as a counterstain.

Biopsy material was used as positive controls. Negative controls were stained as described above but omitting the first antibody.

Western blot: To test for the possible presence of p53 protein, not detected by IHC, western blot on frozen brain tissue was performed.

Paper V

Immunohistochemistry (IHC): Paraffin embedded brain tissue (autopsy material from three secondary progressive MS cases and one control patient without neurological disease) was stained with polyclonal peroxidase labelled rabbit antihuman IgG, IgM and IgA antibodies.

Bound antibodies were visualized using LSAB peroxidase complex. Mayer or Luxol Fast Blue was used as counterstain.

Direct labelling of human CSF antibodies with fluorescence was performed using Zenon kit (Molecular Probes).The binding pattern was studied in a fluorescence microscope.

Western blot was performed on homogenized frozen brain tissue (autopsy material from two secondary progressive MS cases and one control) with CSF, from three relapsing remitting MS patients, two patients with ON and one control, as primary antibody. Alkaline phosphatase-conjugated rabbit antihuman IgG was used as secondary antibody.

Immunoprecipitation: Tissue extracts for immunoprecipitation were prepared as described for western blots, but diluted in RIPA buffer. CSF was added and the sample rotated over night at 4^{o}C. Protein-A agarose beads were added, the sample rotated and the beads washed and collected by centrifugation. The supernatant was carefully removed and western buffer was added to the beads.

When CSF samples were analysed without tissue extracts, CSF was added directly to the beads, diluted in RIPA buffer and treated as the tissue extracts above.

The samples were incubated and shaken at 70°C followed by centrifugation. The supernatants were collected and loaded at precast 4-12% Bis-Tris gels. After electrophoresis the gels were stained with Coomassie Brilliant Blue. All visible bands were cut out from the gels and protein contents were identified by MALDI-TOF technique at the Swegene proteomics facility at the Gothenburg University.

Results

Investigation of a possible genetic linkage between a polymorphic loci in, or close to the IFNA/B gene, and multiple sclerosis in a familial material (Paper I)

A two-point linkage analysis using the MLINK program was performed on the family material. The model applied for analyses was autosomal dominant inheritance and several different levels of penetrance were tested. No evidence of linkage was found between the IFNA/B locus and MS. In this paper the family material was also analysed with an affected sib-pair method using the Extended Sib-Pair Analysis (ESPA) program.[137] Evidence for increased sharing of alleles, identical by descent (IBD) was not detected.

Investigation of a possible genetic linkage between polymorphic loci in, or close to the IFNG, IFNGR, IFNAR, JAK1, STAT1 and STAT3 genes, and multiple sclerosis in a family material (Paper II)

Candidate gene linkage was analysed by the transmission disequilibrium test (TDT), combined with the Sib-TDT in order to make use also of the family data where parental genotypes were missing but genotypes of unaffected siblings were available.[138] A nonparametric linkage analysis (NPL) was also carried out. However, neither of the analysis revealed significant linkage between the tested IFN system or JAK-STAT pathway genes and MS.

Serological differences in monozygotic twin pairs discordant for multiple sclerosis (Paper III)

We could not identify any common factor present only in the affected twins but differences were noted in serum titers against some neurotropic microorganisms. One MS affected twin had an IgG titer against *Toxoplasma gondii* not found in the healthy twin. In another twin-pair, only the MS affected twin had an IgM titer against *Borrelia burgdorferi*. In general the serum titers were strikingly similar in the MS-affected and nonaffected twins, indicating that MS is not associated with major disturbances of the humoral immune system. However, compared with the population in general, both the affected and unaffected twin in two of the twin pairs showed poor response to vaccination

against mumps. One MS affected twin had no neutralizing antibodies against polio type 3 and low levels against types 1 and 2 despite repeated vaccinations. This may indicate impaired responsiveness to these immunizations.

Immunohistochemical analysis of MxA, p53 and CHOP expression in brain tissue from patients with multiple sclerosis (Paper IV)

Cytoplasmic staining was not detected in anti-MxA stained sections from brain tissue of MS patients or controls obtained by autopsy. The positive control was hepatitis C infected liver sections which showed staining as expected.

The p53 and CHOP antibodies showed no nuclear staining in brain tissue from the MS patients or controls. In contrast, p53 and CHOP stained the positive control sections from breast cancer and myxoid liposarcoma.

A western blot analysis on frozen tissue from MS brain, confirmed the negative p53 IHC results.

CD3 stained a small number of lymphocytes around and inside the vessels in all the MS sections. There was almost no lymphocyte staining in the control brain tissue. Tonsil tissue was used as a positive control and showed intense CD3 staining.

In all MS sections, but not in control brain tissue, all antibodies showed a prominent nonspecific staining along the outermost part of the myelin sheaths in the border region close to plaques. The staining faded with the distance from the plaque and was absent in tissue that appeared normal.

Control staining experiments in which the primary antibody was omitted showed no staining.

Identification of target structures for autoantibodies in brain tissue from patients with multiple sclerosis (Paper V)

Immunohistochemistry (ICH): Staining of autopsy brain tissue from MS patients with anti-IgG revealed a distinct binding pattern along many of the fibres in the plaque margins. The number of stained fibres decreased with the distance from the plaque. Anti-IgM and anti-

IgA showed no, or very weak staining, with similar location to that of anti-IgG. Control sections showed no staining.
Anti-IgG staining of fibres was slightly enhanced in sections that had been preincubated with CSF from MS patients. Control sections incubated with CSF from MS patients failed to show any staining of fibres or other structures.
Direct fluorescence labelling of human CSF antibodies from an MS patient showed similar binding pattern as with anti-IgG.
Anti-IgG stained numerous plasma cells in brain tissue from MS patients. They were seen most frequently around blood vessels. Plasma cells were not visible in control tissue.
Combined staining with anti–IgG and Luxol Fast Blue staining of myelin, revealed that the IgG was bound to structures distinct from myelin, presumably the axons. Bound IgG was observed both in myelinated axons and in totally or partially demyelinated axons.
Figure 2.

Western blot: Extracts from frozen autopsy brain tissue from MS patients and a control were analysed by western blot using CSF from MS patients, patients with ON or healthy controls as primary antibody.
No bands specific for MS tissue were found with any of the CSF antibodies.
An 85 kDa band was detected in an extract from control brain tissue with two MS derived CSF antibodies. This band was not visible in extracts from MS brain.

IP: Immunoprecipitation of extracts from control and MS affected brain tissue was performed with CSF from MS patients as antibody. When size-separated by PAGE and stained with Coomassie Brilliant Blue, there were no differences in the separation pattern of the immunoprecipitates between the two extracts. All visible bands were cut out and their protein content characterized by MALDI-TOF technique. All bands contained different fragments of IgG.

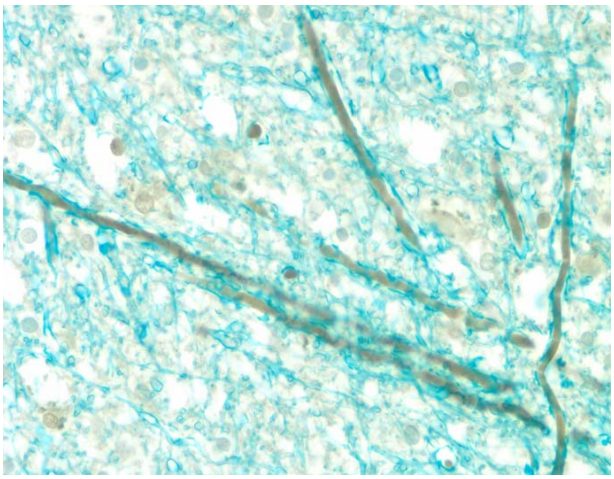

Figure 2 Combined staining with anti-IgG and Luxol Fast Blue staining of myelin in plaque region.

Discussion

The aetiology of MS is unknown but probably results from interplay between as yet unidentified environmental factors and susceptibility genes. In this thesis we have investigated some aspects of the IFN system in MS and also searched for infectious agents in the context of MS aetiology. Furthermore, we have tried to identify target structures for autoantibodies in brain tissue from patients with MS.

The IFNs are a large family of multifunctional secreted proteins involved in, among other things, antiviral defence and immune activation, both believed to be important in MS. The clinical observation that viral infections frequently are associated with or followed by an acute MS relapse suggests that infectious agents may represent an environmental component of the induction and progression of the disease.[139, 140] Furthermore, seasonal variation of IFNG production in multiple sclerosis has been reported, suggesting a potential environmental link between IFNG production and disease pathogenesis and progression.[141] The importance of the IFN system in the outcome of a viral infection is demonstrated by the impaired resistance against different viruses in mice deficient for the receptors IFNAR-2 and IFNGR.[142]

Both the antiviral and the immunomodulatory effects may indicate that the IFN system is involved in the development of MS. The highly polymorphic nature of the genes in the IFN system made them interesting as candidate genetic factors. In paper I and II we searched for linkage between MS and components of the IFN system and the JAK-STAT pathway. However, no linkage was found. A 124bp IFNG allele, in a previous study reported to have a weak association with MS in a Swedish population,[143] was found to be more common than expected among affected individuals, but after adjusting for multiple testing the difference was not significant. To investigate whether alleles at a polymorphic IFNG locus in intron 1 influence subsceptibility to MS, Dai *et al*. performed both nonparametric linkage analysis and transmission disequilibrium testing of 100 nordic sibling pairs, with negative results.[144] In agreement with this, Schrijver *et al*. reported no significant differences in the distribution of IFNG, IFNGR1 or IFNGR2 genotype and allelic frequencies between 509 patients with MS and 109 healthy controls.[145]

We believe, that if there had been a strong linkage between any of the investigated IFN loci and MS, this would have been detected in our studies. More likely several genes, each contributing to a small extent, are involved in the susceptibility to MS and therefore the small number of patients limits the power of our studies. Simultaneous analysis of related genes could improve the power to detect small effects.

During the years several studies have been performed utilising either population-based (case control) or family-based (transmission disequilibrium test) approaches to study candidate genes. The results have been disappointing and no single MS candidate gene has been found. Numerous association and linkage studies have implicated the major histocompatibility complex (MHC) as one component of the genetic aetiology, but additional loci remain to be identified. Systematic search aimed at identifying non-HLA susceptibility genes has been undertaken in several populations by means of linkage studies with microsatellite markers distributed across the whole genome. The conclusion of these studies was that there is no major MS locus, and genetic susceptibility to the disease is most likely explained by the presence of different genes each contributing to the overall familial aggregation.[5] Large scale screenings are needed to identify genetic determinants with a low phenotypic effect. Today collaborative studies screen several thousands of patients with MS with thousands of genetic markers using DNA pooling procedures.

Approximately 75% of twins with an affected identical twin do not develop MS, [37] indicating that environmental factors contribute to the development of the disease. Since monozygotic twins are genetically identical they are ideal in searching for triggering events. In paper III we investigated 3 monozygotic twin pairs discordant for MS for serological differences regarding 21 viruses, 4 bacteria and *Toxoplasma gondii*. We also collected medical and social histories by questionnaires including infectious and other diseases, allergies, vaccinations, exposure to animals, smoking habits, occupational and chemical exposures and travelling to other countries. Although the unaffected twins are young and will be at risk for many more years, at the time of the investigation they were all healthy, indicating that they had not yet been exposed to the environmental factor that triggered the onset of the disease in the affected twin. The serological analyses revealed a previous infection with *Borrelia Burgdorferi* respective *Toxoplasma gondii* in two MS

affected twins, not found in the unaffected twin. There is no evidence that Toxoplasma or Borrelia causes MS in the patients but it cannot be excluded that these pathogens could have acted as precipitating factors. Previous studies investigating a connection between Borrelia and MS have been contradictory.[146,147] In this context it is interesting to note that IFNG is reported to be an absolute requirement for resistance against acute acquired infection with *Toxoplasma gondii* and development of toxoplasmic encephalitis during the late stage of infection.[148]

The hypothesis has been put forward previously that MS could be the result of an aberrant immune response perhaps triggered by delayed exposure to common childhood infections.[149, 150] Our study of virus antibody titers against many common childhood diseases did not support this. The titers were strikingly similar between the healthy and MS affected twins indicating that MS is not associated with major disturbances of the humoral immune system. This is in agreement with a recently published large Danish investigation reporting that measles, rubella, mumps, varicella, pertussis and scarlet fever, even if aquired late in childhood, were not associated with increased risk of MS later in life.[151]

In our study all six twins had received repeated polio vaccinations as part of the common childhood immunization plan, yet one MS affected twin had no neutralizing antibodies against polio type 3 and low levels against types 1 and 2. However when he was revaccinated recently he responded with normal antibody titers showing that presently he has a functional humoral immune system. The reason for the poor response previously is unknown. It is possible that concomitant infections inhibited the immunization.

In this study both twins in two of three tested twin pairs had an impaired responsiveness to vaccination against mumps. The significance of this is observation is, unclear however, since the mumps vaccine was reported to be less efficient (Dr T Dalianis personal communication).

Several common childhood infections have been proposed to be involved in MS but this hypothesis was not supported by the findings in our study. Furthermore the vaccination programs against these diseases have not yet changed the incidence of MS in young adults.

Many types of viruses induce interferon production, but it is not known whether interferon is produced in the MS lesions. MxA is a protein induced specifically by IFNA/B. In

patients with MS who are treated with IFNB, high levels of anti-IFNB antibodies results in loss of the MxA response and this is a sensitive marker of lost bioactivity.[88] We did not detect anti-MxA cytoplasmic staining of cellbodies in brain tissue from secondary progressive MS patients or controls. This is in agreement with a previous study where MxA protein expression was not detected in brain tissue from six MS patients, but was detected in all HIV patients with proven opportunistic viral encephalitis (CMV encephalitis 5/5, PML 9/9) and other patients suffering from viral encephalitis (HSV encephalitis 1/1, Rabies encephalitis 1/1, SSPE 2/2).[152] Therefore MxA and consequently IFNA/B, are probably not produced in lesions from secondary progressive MS. If local virus infections are involved in the lesion formation, they seem to fail to induce an IFN response.

Many MS studies have reported damage to the myelin producing oligodendrocytes by immune or toxic mechanisms.[153] In our analysis of the expression of the stress-induced proteins, p53 and CHOP, we did not detect nuclear staining and therefore no support for the presence of these proteins in brain tissue from secondary progressive MS patients. Previous studies regarding p53 expression in demyelinating diseases have yielded conflicting results.[102, 110, 109] However, our western blot analysis on frozen brain tissue confirmed the negative p53 IHC results, and support the conclusion that there is no general cellular stress response induced in secondary progressive MS.

The oligoclonal bands detected in the CSF of MS patients are believed to be the result of immunization against unknown antigens. Assuming that the antibodies in the CSF of MS patients should bind to their antigens *in vivo,* we attempted to identify structures in MS brain tissue that contained the patient's own antibodies, using IHC methods.

Brain tissue from secondary progressive MS patients contained a unique structure that bound IgG antibodies from the patients. Pretreatment of these brain sections with CSF from relapsing remitting MS patients enhanced the anti-IgG staining. This was not seen in control brain tissue. Direct fluorescence labelled human CSF antibodies from MS patients showed similar binding pattern as anti-IgG in brain tissue from secondary progressive MS patients. Fluorescence labelled antibodies did not bind to control brain tissue. The results indicate that the antigens/target structures are not present in normal brain.

Double staining showed that the IgG was bound to structures distinct from myelin, presumably axons. We observed this both in myelinated axons and in partially or totally demyelinated axons. This suggests that the antibody binding may be an early event in myelin and axon destruction.

We failed to detect MS specific antigens by western blot and immune precipitation. This could depend on very low amounts of the antigen(s) being present. Alternatively, the antigen(s) may have been insoluble under the extraction conditions used or they may be of non-protein nature and therefore undetectable with the methods we used.

Populärvetenskaplig sammanfattning.

Multipel skleros (MS) är en vanlig orsak till neurologiskt handikapp hos unga vuxna. I Sverige insjuknar drygt 1:1000 i MS, kvinnor dubbelt så ofta som män.

Det är en kronisk sjukdom i centrala nervsystemet karakteriserad av multipla skador i framför allt den vita substansen i hjärnan och ryggmärgen. Isoleringen (myelinet) runt nervtrådarna bryts ner och nervfibern (axonet) kan inte fortleda impulser på normalt sätt. Symtomen beror på vilken del av nervsystemet som drabbas, vanliga symtom är kraftnedsättning, koordinationsstörning, känselnedsättning, synstörning, påverkan på urinblåsan samt tilltagande gångstörning.

Initialt uppträder symtomen oftast i skov, perioder av symtom från nervsystemet, omväxlande med symtomfria perioder. Prognosen är oviss. Den kan ha ett godartat förlopp men hos majoriteten av de drabbade övergår sjukdomen efter en tid i en sekundär progressiv fas med gradvis försämring. I ett mindre antal fall är sjukdomen progressiv från början.

Orsaken till MS är inte klarlagd men man tror att okända miljöfaktorer, t.ex virus, kan utlösa sjukdomen hos individer med en ärftlig predisposition.

Det finns ingen botande behandling, men under de senaste 10 åren har man kunnat bromsa förloppet av den skovvis förlöpande formen av MS genom behandling med interferon beta. Interferon beta är ett protein som gör celler mindre känsliga mot virusinfektioner men som också har andra effekter på immunförsvaret. I behandlingen av MS betonar man framför allt de immunomodulerande egenskaperna hos interferon beta, så som den inflammationsdämpande effekten i nervsystemet. Man har också gjort behandlingsförsök med en annan typ av interferon, interferon gamma, som emellertid försämrade sjukdomen. Verkningsmekanismen hos interferon gamma tycks i flera avseenden vara motsatt den av interferon beta med bl.a en inflammationsökande effekt.

Det har i flera studier rapporterats att MS patienter har ett onormalt svar på virusinfektioner, men också att interferonproduktionen är avvikande.

Generna för interferonerna och deras receptorer finns i olika varianter (alleler) hos olika människor. I arbete I och II undersökte vi om det fanns ett samband mellan MS och

nedärvningen av någon speciell allel i vissa komponenter av interferonsystemet. Vi utgick från ett familjematerial där minst två i varje familj hade MS och undersökte DNA från perifiert blod med hjälp av PCR metodik. Inget signifikant samband kunde fastställas mellan någon av dessa interferon loci och MS.

Enäggstvillingar där enbart den ena har MS, erbjuder en möjlighet att kartlägga om två individer med identisk genetisk bakgrund och sannolikt mycket snarlik uppväxtmiljö uppvisar samma reaktionsmönster mot vanliga virus och andra infektiösa agens. I arbete III undersökte vi 3 sådana tvillingpar avseende antikroppssvar mot 21 virus, 4 bakterier och *Toxoplasma gondii*. Två MS sjuka tvillingar, men inte de friska tvillingsyskonen visade tecken på genomgången infektion med Borrelia respektive Toxoplasma, båda neurotropa agens. I stort var dock antikroppstitrarna påfallande lika hos de friska och sjuka tvillingarna, vilket talar emot en allvarlig störning i det humorala immunförsvaret.

I arbete IV försöker vi kartlägga om det finns tecken på interferonproduktion, och därmed också misstanke på virusinfektion, i skadeområden i hjärnvävnad från nyligen avlidna patienter med sekundär progressiv MS. Eftersom interferon inte upplagras, utan utsöndras snabbt från virusinfekterade celler, har det varit svårt att märka in dessa celler med antikroppar mot interferon. För att lösa problemet använde vi istället antikroppar mot det interferon inducerade proteinet MxA. Detta är ett cytoplasmatiskt protein som hindrar förökningen av många virus. Hos MS patienter som behandlas med interferon beta mäter man ofta nivån av detta protein som ett mått på behandlingseffekten.

Vi kunde inte påvisa MxA, och därmed inte heller interferonproduktion, i den undersökta hjärnvävnaden från MS patienter.

Normalt bildas inga antikroppar i centrala nervsystemet. I samband med inflammationen vid MS kan antikroppsproducerande celler ta sig igenom blod-hjärn barriären. Antikroppar upptäcks i ryggmärgsvätskan hos 90-95 % av alla MS patienter och utgör ett diagnostiskt hjälpmedel. Man har inte kunnat fastställa vad antikropparna är riktade mot.

I avhandlingens sista arbete undersöker vi med immunohistokemiska metoder om det finns ”MS-specifika” antigen i skadeområdena i hjärnvävnaden från MS patienter, genom att använda IgG specifika antikroppar, och ryggmärgsvätska från MS patienter som antikropp.

Vi fann antikroppar bundna på axonen i skadeområdenas randzon hos MS patienterna, men inte i kontrollhjärnvävnad. Denna bindning av antikroppar accentuerades när vävnaden förbehandlats med ryggmärgsvätska från MS patienter. Antikropparna påvisades både på myeliniserade axon och på helt eller delvis demyeliniserade axon något som talar för att antikroppsbildningen kan vara ett tidigt fenomen i sjukdomsförloppet.

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Doubt is not a pleasant condition
but certainty is absurd
Voltaire