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Studies on etiology and pathogenesis of multiple sclerosis

PhD thesis

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Abstract

An initial study in this thesis concerned the epidemiology of multiple sclerosis (MS) to provide a background for future clinical studies of MS patients. The cerebrospinal fluid bank was up-dated for similar reasons. Our epidemiological results were comparable to those found in the literature. The etiology of MS is still unknown. Environmental as well as genetic factors seem to be involved. Latent neurotrop human herpes virus 6 type A was more often detected in the CSF of MS patients suggesting an involvement in the initialization or progression of the disease. Genetic susceptibility of MS is presumed, but the genes involved have not been identified thus broad population based studies are needed to find the regions of interest. In an international survey 33 potential markers were found in the Hungarian population. Reporting rare cases of familial aggregation could provide additional data to the genetic susceptibility. We reported on three affected sisters and their healthy parents and grandparents. As evidence is piling up, that nervous and the immune system communicate using the sympathetic nervous system and the hypothalamo-pituitary-adrenal gland axis as integrative interfaces, catecholamines and their role as a transmitter in both systems become more interesting in autoimmune diseases, such as MS. We found decreased norepinephrine levels in stable MS patients, while first-attack MS patients had higher epinephrine levels, suggesting an active immune system in those patients. In an open-labeled trial on long-term interferon-beta administration, sustained decrease in relapse rate were found, and in line with this finding, the days of hospitalization and steroid need during the year was also decreased.

Original papers related to the thesis

Paper I - Bencsik K, **Rajda C**, Klivényi P, Járdánházy T, Vécsei L. The prevalence of multiple sclerosis in the Hungarian city of Szeged. Acta Neurol Scand 1998;97:315-319.

Paper II - Bencsik K, **Rajda C,** Füvesi J, Klivényi P, Járdánházy T, Török M, Vécsei L. The prevalence of multiple sclerosis, distribution of clinical forms of the disease and functional status of patients in Csongrád County, Hungary. Eur Neurol 2001;46:206-209.

Paper III - Ongrádi J, **Rajda C,** Maródi CL, Csiszár A, Vécsei L. A pilot study on the antibodies to HHV-6 variants and HHV-7 in CSF of MS patients. J Neurovirology 1999;5:529-532.

Paper IV - Rajda C, Bencsik K, Seres E, Jonasdottir A, Foltynie T, Sawcer S, Benediktsson K, Fossdal R, Setakis E, Compston A, Vécsei, L. A genome-wide screen for association in Hungarian multiple sclerosis. J Neuroimmunol 2003;143:87-84.

Paper V Bencsik K, **Rajda C**, Seres E, Vörös E, Janáky M, Dibó Gy, Járdánházy T, Vécsei L. Familial multiple sclerosis: case study of three affected siblings. Acta Neurol Scand 2002:106:392-395.

Paper VI - **Rajda C**, Bencsik K, Vécsei L, Bergquist J. Intracellular catecholamine contents of peripheral blood lymphocytes in multiple sclerosis. J Neuroimmunol 2002;124:93-100.

Paper VII - Bencsik K, **Rajda C**, Füvesi J, Járdánházy T, Török M, Vécsei L. Experiences with interferon-beta-1b treatment in MS after three year follow-up. Swiss Med Wkly 2002;132:237.

Book chapter and abstracts related to the thesis

Rajda C, Bencsik K, Klivényi P, Seres E, Vécsei L. Patokémiai változások szerepe a sclerosis multiplex kialakulásában (The role of pathochemical alterations in the genesis of multiple sclerosis). In: Vécsei L, Komoly S (eds.). Sclerosis multiplex (Multiple sclerosis). 2003:31-45 *(in Hungarian)*.

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Rajda C, Bencsik K, Török M, Vécsei L. Experiences with mitoxantrone treatment – side effects in secondary progressive multiple sclerosis patients. Mult Scler 2002;8:161.

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Rajda C, Bencsik K, Seres E, Klivényi P, Vécsei L, Bergquist J. Peptidek és katekolaminok sclerosis multiplexben (Peptides and catecholamines in multiple sclerosis). Liquordiagnostica 2001: 7 (in Hungarian).

Rajda C, Bencsik K, Seres E, Vécsei L, Bergquist J. Intracelluláris noradrenalin szint monitorozás sclerosis multiplexes betegek limfocitáiban (Monitoring the intracellular norepinephrine level in the lymphocytes of multiple sclerosis patients). Fiatal Neurológusok Fóruma Budapest, 2001: 28 (in Hungarian).

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List of abbreviation

AIP – allele image profile

BBB – blood-brain barrier

CD – cluster designation

CNS – central nervous system

CSF – cerebrospinal fluid

DA - dopamine

DNA – desoxyribonucleic acid

DOPAC - dihydroxyphenylacetic acid

E - epinephrine

EAE – experimental allergic encephalomyelitis

ELISA – enzyme-linked immunosorbent assay

GAMES – Genetic Analysis of Multiple Sclerosis in EuropeanS

HHV-6 – human herpes virus 6

HHV-7 – human herpes virus 7

HPA – hypothalamus-pituitary gland-adrenal medulla axis

IFN – interferon

IL- interleukin

IgG – immunoglobulin G

L-DOPA - L-hydroxylphenylalanine

LN – laser nephelometry

MAb – monoclonal antibody

MES - 2-N-morpholino ethanosulfonic acid

MHC- major histocompatibility complex

MRI – magnetic resonance imaging

MS- multiple sclerosis

NE - norepinephrine

NF-κB – nuclear factor-κB

NK – natural killer cell

NOS – nitric oxyde synthase

OCB – oligoclonal bands

PCR – polymerase chain reaction

RR - relapsing remitting

PP - primary progressive

SEM – standard error of mean

SP – secondary progressive

TGFβ – transforming growth factor β

Th – T helper cell

TNF-α – tumor necrosis factor α

UA – uric acid

VEP –visual evoked potential

1. Introduction

1.1 Historical notes on MS

One of the first recorded clinical case of MS originates from Auguste d'Este, Grandson of George III of England (1794-1848), but other cases are also cited, as the case of St. Lidwine of Schiedam and an Icelandic Viking woman named "Halla" from the 14th century. Other diaries from the 1800s and 1900s are that of the poet Heinrich Heine and Barbellion (as reviewed by Butler and Bennett) (1). The first pathoanatomical description originates from Robert Carswell (1838) and Jean Cruvelhier from 1842. The exact description of the clinical symptoms and histological signs of MS are from Jean Martin Charcot (1825-1893) and M. Vulpian described in the year 1868 (2), followed by the work of other prominent neurologists, as e.g. Babinski, who wrote his thesis on MS (3).

1.2 Nomenclature

MS is also known as disseminated sclerosis in the historical literature. It belongs to the group leukoencephalitis. This name points to the disorder mainly affecting the white matter. Other diseases from this group are: neuromyelitis optica (Devic's disease), which is considered to be the extreme form of MS, encephalomyelitis disseminata, parainfectious, postvaccinatious encephalomyelitis, Schilder's encephalitis periaxialis diffusa and Baló's sclerosis concentrica (4). In 1996, a uniform nomenclature was introduced for the clinical course of this disease. These clinical forms are: benign, relapsing-remitting (RR), secondary progressive (SP), relapsing-progressive, primary progressive (PP) (5).

1.3 Epidemiology

MS is the most common neurological disorder in young adults; the age of onset is usually between 20-40 years. The female:male ratio is 2:1, depending on the age of the onset. A distinction between low, medium and high-risk factor areas can be made on the basis of geographical lines of latitude. The disease is most prevalent in high northern latitudes However, the new prevalence tests reveal that in both low and medium-risk factor areas there can in fact be an enhanced occurrence (6-8). In research on the etiologic factors of MS, the occurrence and clinical forms of the disease have been examined in different population groups, such as Mestizos, Indians, etc. (6, 9). The Gypsy minority race, just like the Lapps of Scandinavia, the North American Indians of Canada, the Afro-Americans, and the Maoris of New Zealand, have been found to be resistant against multiple sclerosis (10-15). In Hungary, Pálffy et al. (10) calculated the prevalence in Baranya County in 1983, which was 37/100,000. The prevalence in Fejér County in Hungary in 1992 was found by Guseo et al. (16) to be 69/100,000. Unfortunately, some of those patients did not undergo MRI examinations. Dean (17) determined the prevalence of multiple sclerosis within the continental zone as 30-80/100,000. The occurrence was found to be related to geographical distribution, migration and genetic contribution (6, 18-23).

1.4 Etiology and pathogenesis

Multiple sclerosis is an autoimmune demyelinating disease, where inflammation and axonal loss takes place at the same time (24, 25). The disease is characterized by inflammatory lesions in the white matter of the nervous system, consisting of a specific immune response to the myelin sheet. In MS, axonal loss is present in the pathogenesis of the disease at early stages (26, 27). Current hypotheses on the pathogenesis of MS suggest that the primary peripheral activation of autoreactive T helper-1 lymphocytes precede the

recognition of central nervous system (CNS) auto-antigens. These T cells proliferate, secrete cytokines and cross the blood brain barrier (BBB) to find their antigens in the CNS where they cause further inflammatory damage. It has been hypothesized that RR MS is driven by a systemic antigen presentation and that chronic progressive MS depends on the CNS presentation of antigens (28). Recent reports of studies point to an immunopathogenetic heterogeneity, where the patterns of demyelination were heterogeneous between patients, but homogenous within the plaques from the same patient (29). Four patterns of demyelination in MS were found: Pattern I is the macrophage associated demyelination, Pattern II is characterized by antibody mediated demyelination, mechanism responsible for Pattern III results in distal oligodendropathy and Pattern IV is primary oligodendrocyte degeneration. Pattern I closely resembles myelin destruction in mouse models of EAE. All patients with Devic's type neuromyelitis optica had Pattern II, Pattern III is commonly found in virus-induced human white-matter diseases and Balo's type concentric lesions, while Pattern IV have been found in a small subset of PP MS patients (as reviewed by Lassmann) (30).

The etiology of MS is still not clear. Environmental, as well as genetic factors seem to be implicated in the pathogenesis of the disease.

1.4.1. Environmental factors - Infectious theories

The hypotheses of infectious involvement are based on the seasonal variation in the incidence of new MS cases, the geographic association of disease susceptibility with evidence of MS clustering, and the idea that anti-myelin T cells or autoantibodies could be elicited by molecular mimicry (as reviewed by Swanborg et al.) (31). Viral theories are based on epidemiological evidence of childhood exposure to infectious agents, the increase in disease exacerbations with viral infection, evidence that migration to and from high-risk

areas influences the likelihood of developing MS, abnormal immune responses to a variety of viruses, and the analogy with animal models and other human diseases in which viruses can cause diseases with long incubation periods, a RR course, and demyelination (as reviewed by Soldan et al.) (32). Many of these studies involve the demonstration of increased antibody titers to a particular virus, whereas some describe isolation of virus from MS material. However, no virus to date has been definitively associated with this disease. Human herpesvirus type 6 (HHV-6) is a β-herpes virus isolated initially from peripheral blood mononuclear cells of infected AIDS patients (32). There is both serological and molecular evidence that, HHV-6 might have a role in the pathogenesis of multiple sclerosis (MS). Isolates of HHV-6 are grouped as variants A and B, and the contradictory results using different serological and molecular assays (32, 33) raises the possibility that HHV-6A and HHV-6B might exert different effects in disease progression. Recent reports indicate that the two variants have distinct biological properties and pathogenic potentials. In an individual no cross-immunity exists between HHV-6A and HHV-6B despite their genomic similarity (34). In spite of the integrity of the BBB against HHV-7 (35), both HHV-6A (34) and HHV-6B (36) may reach the central nervous system (CNS). In children and adults with dual infection, only HHV-6A persisted in CSF, which suggested that HHV-6A had greater neurotropism, but HHV-6B tended to be more prominent in other tissues (34, 37). In contrast, the presence of HHV-6B sequences was found to be common in the brains from MS patients and controls, but the high degree expression of HHV-6B in the plaque-associated oligodendrocytes (to be destructed) suggests that rather local instead of general effects of viruses contribute to MS pathogenesis (36).

1.4.2. Genetic factors

One of the underlying cause of autoimmune diseases is the inheritance of sets of genetic components that individually form part of a functioning immune system but that, when combined, increase the chances of susceptibility to an autoimmune disorder (38). As results of modern genetic studies it became evident, that MS results from an interaction of genetic and nongenetic factors. Familial aggregation of MS is due to the genetic material these relatives share with the individual having MS, the disease appears to be oligogenic. HLA is not the deterministic gene, and genetic susceptibility factors may overlap, at least to some extent, among the general population and individuals having MS (19). Age adjusted family risks for MS were greater than the lifetime prevalence for the general population (39). Study of adopted and adoptive parents indicated that the excess of MS among relatives is dependent upon the sharing of genetic material (40). These findings were followed by molecular genetic studies for candidate genes. The only consistently positive finding has been for the major histocompatibility complex (MHC) located on chromosome 6p21 (as reviewed by Sadovnick) (19).

1.5. Neuroimmunological interaction

1.5.1. Interaction between the nervous and immune system

During the immune response the CNS and the immune system communicate using the sympathetic nervous system and the HPA axis as integrative interfaces (41). Norepinephrine fulfills the criteria for a neurotransmitter in the lymphoid organs, which are receiving an extensive sympathetic innervation, and the target immune cells express adrenoreceptors. Through stimulation of these receptors, locally released or circulating catecholamines affect lymphocyte traffic and proliferation. Norepinephrine and epinephrine inhibit the Th1 (pro-inflammatory) type cytokine production and stimulate the

Th2 (anti-inflammatory) type cytokine production (as reviewed by Elenkov et al. 2000 and Sanders and Straub, 2002) (41, 42).

1.5.2. Beta-adrenergic receptors

The effect of catecholamines, secreted by the sympathetic nervous system, predominantly acts on human T cells of the $CD8^+$, $CD28^-$ (suppressor) subset (43). This subset also has the highest β-adrenergic receptor density. Antigen presenting cells in the CNS and lymphocytes bear primarily β-adrenergic receptors. The lymphocytes of relapsing-remitting MS patients (44), SP patients (43) and PP MS patients (45) express more β-adrenergic receptors. Both EAE and human studies points to altered β-adrenerg receptor function in MS. Beta-adrenoreceptor agonists has been reported to suppress EAE symptoms (46, 47), while normal appearing white matter and plaques in MS patients are lacking β2-adrenerg receptors, which were present in the white matter of control brain (48, 49). Catecholamines also affect NK cell function through β-adrenergic receptors (50).

1.5.3. Catecholamines in MS

Lymphocytes are actively involved in the synthesis, uptake and degradation of catecholamines (51, 52). Furthermore, catecholamines have been found inside the nuclear envelope (51), suggesting a possible direct interaction with the transcription machinery or via an interaction with the NF-κB regulatory system (53). Recent results suggest a crucial role of NF-κB1 in the activation and differentiation of autoreactive T cells. Blocking NF-κB function can be an effective way to prevent autoimmune encephalomyelitis (54). Elevated regional levels of catecholamines might lead to suppression and finally apoptosis that could partly explain the immune privilege of the brain (55). Sophisticated analytical methods made possible to measure the amount of catecholamines in a single lymphocyte in

the CNS (56) in a picomole level (10^{-12} mol/cell) (51). Polymorphisms of the gene encoding the terminal enzyme of the catecholaminergic biosynthesis pathway (phenylethanolamine N-methyltransferase) shows association with MS (57).

1.6. Clinical features of MS

As the name of the disease indicates, the clinical symptoms are also multiple: disturbances in the visual, motor, sensory and cognitive function. In 2001, an international panel revised the Poser's diagnostic criteria (58), the new criteria is known as McDonald's diagnostic criteria (59), which integrated the magnetic resonance imaging (MRI) with clinical and other paraclinical diagnostic methods, and defined the criteria of the clinically isolated syndromes and primary progressive form more precisely. The focus still remained on the objective demonstration of dissemination of lesions in both time and space.

1.7. Magnetic resonance imaging (MRI)

With the help of the more sophisticated diagnostic tests (e.g. MRI) the diagnosis of MS became easier. The hallmark of MS on MR images are the T2 weighted lesions, on T1 scans the black holes and the Gadolinium enhancement in active lesions, brain atrophy has been detected at early stages (as reviewed by Miller et al., 1998) (60).

1.8. CSF findings

Cell count in MS is usually normal, or slightly elevated, pleiocytosis is also reported. The CSF cytology has not a typical appearance. In the active phase activated lymphocytes can be found. The CSF protein concentrations are often increased, and the blood-CSF barrier is many times damaged. Elevated albumin ratio, total protein, gamma globulin level and pathological IgG index is often found (61, 62). A relation between age and protein levels in

the CSF was found in healthy individuals (63). The gammaglobulins in the CSF migrate in agarose electrophoresis as abnormal discrete populations, the oligoclonal bands (OCB) (64). These bands were seen in 95% of the patients with isoelectric focusing (65). Quantitative measurements of IgG production in the CNS are less sensitive in the diagnosis of MS, than isoelectric focusing.

1.9. The interferonβ treatment in MS

Beta-IFNs are used to treat clinically active MS patients to reduce the relapse rate and decrease the progression of the disease (66, 67). In the mechanism of action immunomodulatory rather than antiviral or antiproliferative effects seem to be operative. Beta-IFNs down regulate the IFN-gamma induced increase in MHC class-II molecules on peripheral blood monocytes and other antigen presenting cells, inhibits monocyte activation and antigen presentation to T cells (see recent review by Milo and Panitch) (68). Beta-IFN inhibits IFN-gamma, TNF-α and lymphotoxin secretion, up-regulation of the anti-inflammatory cytokines TGFβ and IL-10, and suppression of IL-12. These effects all contribute to a shift from Thl to Th2 cell-mediated immune responses. It prevents disruption of the BBB and the migration of lymphocytes into the CNS, as evidenced by the dramatic decline in the number of enhancing MRI lesions in patients with MS who where treated with IFNβ-1b or -1a. Beta-IFN quickly binds to its receptor on target cells and activates interferon-inducible genes that mediate various activities. Some of these gene products, e.g. 2,5-oligoadenylate synthetase, β2-microglobulin and neopterin, serve as biological markers. After single sc. dose of IFNβ-1b, their levels are elevated for more than 3 days. The most often occurring side effects of the β-IFN therapy are flu-like symptoms and depression (68). Despite the clinical advances, the mechanisms by which β-IFN works in MS remain unclear.

Other medications used in MS e.g. glatiramer acetate, intravenous IgG, mitoxantron are not discussed in detail in this thesis.

1.10. Aims of the study

♁- To calculate the prevalence and incidence of MS in the population of Szeged and Csongrád county, to estimate the proportion of the different clinical forms, the relapse rate and their neurological condition. These patients give the major proportion among the patients cared at the MS outpatient Unit at the Department of Neurology, University of Szeged (Paper I and II).

♁-To standardize the conditions of CSF sampling, which samples are later stored at the CSF bank, and provide a CSF bank register, where the decoding and the sample collection for further clinical trials is easy to handle (Paper III).

♁-In the etiology of MS environmental as well as genetic factors are involved. Among the environmental factors virus infections are most often in connection with the first MS relapse. Beside the viruses Ebstein Barr and others, the herpes virus family was found to be involved in the pathology of MS. To show the presence of the latent neurotrop members of the herpes virus family, the HHV-6 and HHV-7 from sera and CSF of MS patents and controls (Paper III).

♁-To study genome-wide screen associations in MS patients in an international survey (Paper IV).

♁-To present a case report of an unusual familial aggregation of MS (Paper V).

♿-To quantify the intracellular catecholamine levels in the peripheral blood lymphocytes of MS patients and controls, presuming that they can be markers of the ongoing immunological activity (Paper VI).

♿-To evaluate the conditions of MS patients after 3-years of IFNβ-1b therapy, regarding relapse rate, progression index, neurological condition and days spent in hospital and amount of steroids needed in a year (Paper VII).

2. Patients and methods

2.1. Patients

2.1.1. Patients and methods in the epidemiological studies of Szeged and Csongrád county (Paper I and II)

In Szeged and the surrounding counties (e. g. Csongrád county) the MS Outpatient Unit, Department of Neurology, University of Szeged deals with the medical care for MS. The number of patients cared at the MS outpatient unit was increasing to the double in the middle of the '90s (approximately 600 patients). We estimated the prevalence, the incidence, the female:male ratio, the clinical forms according the Poser diagnostic criteria (58) and the uniform nomenclature (5), the functional status (69) in Szeged and Csongrád county. The medical records at general practitioners, neurological- and ophtalmological departments and homes for aged were checked for these studies. The epidemiological data were also based on the regularly updated MS register, which is used since 1996. The diagnosis of the patients is based on the MRI and CSF findings, and additionally VEP results. In Paper I, the number of inhabitants in Szeged on January 7, 1997 was 198,682

(Hungarian Central Statistical Office – Központi Statisztikai Hivatal). 130 MS patients from Szeged were cared at the MS Outpatient Unit. In Paper II, according to the files of the Hungarian Central Statistical Office Szeged City (the Country town of Csongrád County) had 198,686 inhabitants, Csongrád County has an additional 222,506 inhabitants. In the study 400,128 inhabitants were the representative sample; the population of a small city with 21,064 residents was excluded because there were no available data.

2.1.2. Patients and controls in the HHV-6 study (Paper III)

The sera-CSF sample pairs were collected from the CSF bank, using the register to find MS patients with abnormal CSF results and age-matched controls. Samples of seven RR MS patients and 6 patients with other neurological diseases were evaluated.

2.1.3. Patients and controls in the GAMES study (Paper IV)

The 88 unrelated MS patients were recruited by the MS Outpatient Unit. The 128 healthy blood donors fulfilled the criteria of the regulations of the local ethical committee and all patients from the cases group met the Poser's criteria (58). The mean age was 35 years in the cases group and the sex was consistent with previous population based series 3.6 femal:1 male. The ratio was 2.6/1 in the unrelated control group. Considering disease course, 90% of the patients had RR disease course, and 10% of patients had SP disease.

2.1.4. Patients and their relatives in the case report (Paper V)

Three sisters suffering from MS and their healthy parents and grandparents were explored. Medical history was taken at the MS Outpatient Unit. Each family member's neurological condition was evaluated. At the time of the report the patients were 24, 26 and 27 years old. The symptoms of the eldest sister began in 1993 with lower-limb weakness and

paraesthesia. Two months after her second labor in March 1998, she was admitted to hospital with limb weakness, nystagmus and ataxia. MRI of the brain, the visual, brainstem and somatosensory evoked potentials, and CSF examinations verified MS. The middle sister exhibited left-side optic neuritis at the initial examinations in February 1998. The MRI findings on the head and optic nerve, the CSF examinations and the electrophysiology all pointed to MS. Despite a negative neurological condition, the third sister had subjective complaints such as paraesthesia of variable intensity in all the extremities and vertigo. The MRI of the brain revealed multiple periventricular T2-weighted lesions, and oligoclonal bands were detected in the CSF by isoelectric focusing. On the Poser diagnostic criteria (58), one of the three sisters was in category B1, and the others in category B2. Both parents and all four grandparents are without neurological signs; the brain MRI examinations on the parents were negative.

2.1.5. Patients and controls in the catecholamine study (Paper VI)

In total 67 patients were examined and were found to have clinically definitive and laboratory-supported definitive MS according to the Poser criteria (58). In the patients group 10 subjects were laboratory-supported definitive (first attack) patients, 48 had relapsing-remitting course MS. Both the CSF findings (oligoclonal bands with isoelectric focusing electrophoresis) and MRI examinations (several periventricular T2-weighted lesions) of the first attack patients supported the MS diagnosis. All the relapsing-remitting patients were in remission, and none of the patients received steroid therapy within 30 days and none of them were on tricyclic antidepressants, cardiac drugs or amantadine. The neurological conditions of the patients were expressed in the Kurtzke expanded disability status scale (EDSS) (69). Healthy individuals (n=19) served as controls. The ethical committee of the Albert Szent-Györgyi Medical School (886/1998) had approved the

study. For statistical analysis patient's subgroups were formed according to the a.) clinical course of the disease: 1st attack (n=10), relapsing-remitting (n=48); b.) EDSS score: EDSS score below (n=49) and above 4.0 (n=9); c.) duration of the disease: less (n=30) and more than 5 years (n=28); d.) time to the last relapse: relapse within 6 months (n=19) and more than 6 months (n=39).

2.1.6. Patients on inteferonβ-1b treatment (Paper VII)

All patients recruited for interferon treatment in between July and November 1996 at the MS outpatient unit were involved in this trial. In line with the guidelines of the American Academy of Neurology (70) 36 patients have been randomized. All the patients were in the laboratory-supported definite MS category according to Poser's diagnostic criteria (58) with oligoclonal bands and MRI findings pointing to MS. The male:female ratio was 10:26. 34 patients had RR and two patients relapsing-progressive course of the disease. 22 females and 9 males completed the study. Patients received 8 MIU IFN-β-1b sc. every other day. EDSS scores have been determined at the beginning of the therapy, monthly and in the case of exacerbations before and after the mega-dose steroid treatment. Laboratory parameters were controlled (routine urine and blood tests, glucose, ions, kidney and liver functional tests) monthly for 3 months, followed by controls every 3rd month. The side effects were recorded monthly. In case of an exacerbation the EDSS score before and after the steroid treatment and one month after the relapse was determined. The steroid needs required to treat relapse and the days of hospitalization were calculated. The relapse-rates concerning the 2 years prior the treatment (1995-96) and the three years of the IFN-β-1b treatment, were compared together with the EDSS scores respectively. The duration of hospitalization, the steroid needs for remissions in 1995-96 and in the three years of

therapy were calculated. Statistical analysis was made by one-way ANOVA analysis followed by pairwise comparison with Bonferroni.

2.2. Methods

2.2.1. Samples from the CSF bank (Paper III)

Emerging need has become evident for a well-documented and updated CSF bank, where the CSF samples are taken at standardized conditions to be comparable. From the end of 1997 centrifuged sera and CSF pairs were labeled with an encoded uniform label before placing it to the –70°C freezer. For decoding a daily updated register is used, containing the cell count, and albumin content. The colleagues at the Department of Neurology, Neurosurgery and Intensive Care Units were informed to take equal amounts of CSF (12 ml recommended) in order to have comparable CSF samples. Previous studies proved a protein gradient in the CSF (71, 72). If the amount differs this should be referred on the laboratory sheet. This sheet was specially developed in 1997 for the faster processing of the CSF from the laboratory to the CSF bank. Instead of handwriting, boxes (e.g. ☑) were of help for the laboratory assistants. The delivery of the CSF occurs immediately after the lumbar puncture followed by a phone call for a technician in medicine. From the lumbar punction until the storage approximately half-hour is consumed, though sensitive molecules in the CSF samples are preserved in this short time period. The CSF was centrifuged at low speed, followed by the quantitation of the leukocyte number in the pellet (Fuchs, Rosenthal chamber). The supernatant was kept for further analysis in the –70°C freezer. The date of the sampling, number of the vials, name, presumed diagnosis and the CSF finding on proteins were registered in the diary.

2.2.2. Cerebrospinal fluid protein analysis (Paper III and V)

The following descriptions of the methods of CSF analysis are routine methods in our laboratory using external control (73, 74). Quantitation of the total protein was performed by trichloroacetic acid precipitation.

2.2.3. Laser nephelometry (LN) (Paper III and V)

The laser scattering method for quantitation of albumin, IgG, IgM and IgA was used (Dosascat, Dosatec GmbH, Munich, Germany) (75, 76). All parameters were determined simultaneously in CSF and diluted sera by LN. LN antisera, standards and control sera were purchased from Dosatec GmbH. Serum dilutions were adapted for CSF calibration curves. IgG, IgA, IgM and albumin were measured according the manufacturer's instructions for CSF samples. Dilution series were used for the standard curves, the curves were modified according the Reiber formula (77).

2.2.4. Agarose gel electrophoresis and isoelectric focusing (Paper III and V)

The proteins in the CSF were analyzed using agarose gel electrophoresis and isoelectric focusing followed by silver staining, as a routine method in the CSF laboratory at the Department of Neurology (78-80). For the detection of IgG bands immunoblot was used (81).

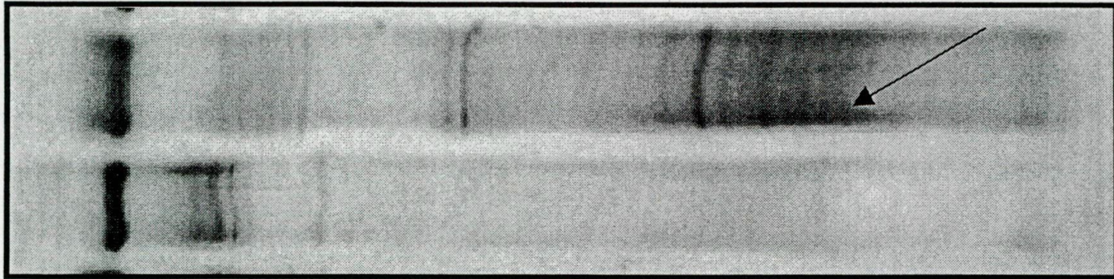

Figure 1. Agarose gel electrophoresis (CSF and sera pair) with oligoclonal bands in the CSF gamma protein region points to intrathecal inflammation in MS. The oligoclonal bands are not present in the sera.

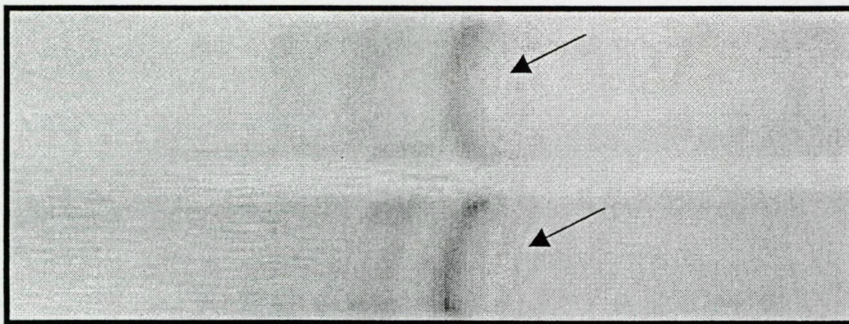

Figure 2. Immunoblotting – detection of IgG oligoclonal bands

2.2.5. ELISA (Paper III)

Sensitive ELISA was used to quantify variant specific IgG and IgM, as described (Maródi et al., 1998). Briefly, JJHAN cells infected with HHV-6A GS, MOLT-3 cells infected with HHV-6B Z29, Sup-T1 cells infected with HHV-7 RK strain served as antigens and were fixed to 96 well polystyrene plates, when OHV-1 monoclonal antibody (MAb; Advanced Biotechnologies Inc., Columbia, MD, US) to both HHV-6 variants and RK-4 MAb to HHV-7 in indirect immunofluorescent assays detected equal ratio (38 to 40%) of cells containing viral antigens. HHV-6 variant specificity was verified by competitive binding of anti-HHV-6B 101k MAb (P.E.Pellett, Atlanta, GA, US). Competing IgG was removed

from aliquots of each serum and CSF by mixing them with Protein-A Sepharose 4B (Sigma, St. Louis, MO, USA) in 10:1 ratio for1h with subsequent low speed centrifugation. Twofold dilutions of sera and CSF (1:100 to 1:6400) were incubated with the antigens in 96 well polystyrene plates in quadruplicate. After vigorous washings, peroxidase conjugated anti-human IgG and IgM (Sigma), respectively, were adsorbed to human polypeptides in parallel wells, finally orthophenyldiamine substrate was introduced in each well. The optical density, read at 492 nm, 150% higher than that of the standard deviation of established seronegative subjects were regarded as positive. Sera of known seropositive individuals (L. Ceccherini-Nelli, Pisa, D. DiLuca, Ferrara, Italy) served as controls. Serum and CSF dilutions at 1:100 were tested in quadruplicate for non-specific binding to uninfected cells, but no reactions were detected.

2.2.6. DNA extraction and pooling (Paper IV)

Genomic DNA was extracted manually using a standard phenol-chloroform method. The concentration was established measuring the optical density twice; only samples with less than 10% difference in these measures were included. Each sample was then diluted to a concentration of 50 ng/uL. Pools (patients and controls) were constructed by combining equal volumes from contributing samples.

2.2.7. Genotyping (Paper IV)

All markers were amplified in each of the 2 pools using standard polymerase chain reaction (PCR) protocol according to the manufactures recommended conditions. PCR products were electrophoresed twice (82) using an Applied Biosystems 3700 DNA Analyser (Applied Biosystems) and sized using Applied Biosystems GENESCAN software (version 3.5). The allele image patterns (AIPs) generated were analyzed using the

perl program ALLELPICKER which was specifically written for the GAMES project. This program avoids the need to inspect the AIPs in GENOTYPER and is thus considerably more efficient. On the other hand the program is critically dependent on the sizing algorithm, such that any error in this can result in erroneus data being collected. In order to identify such errors all AIPs from markers showing significant results were inspected manually with GENOTYPER.

2.2.8. Preparation of Lymphocytes (Paper VI)

Peripheral vein blood samples (12 mL) were prepared by centrifugation at 2500xg for 10 minutes. Lymphocytes were isolated by centrifugation on a Lymphoprep® (Nycomed Pharma, Oslo, Norway) density gradient and after washing and centrifugation steps kept in -80° C until analysis. Lymphocytes were extracted by adding 25 uL perchloric acid (containing 1 mM NaEDTA and 1mM Na_2SO_3) to the pellet and ultrasonicated on ice for 2 minutes using a MSE Soniprep 150 probe. After centrifugation (30 min, 4°C, 35000xg) the supernatant was frozen and stored in -80°C until analysis. The pellet was used for spectrophotometric protein quantitation using bicinchoninic acid protein assay reagent (BCA, Pierce Chemical Company, USA).

2.2.9. Capillary Electrophoresis with Electrochemical Detection (Paper VI, Figure 6)

The capillary electrophoretic system used has earlier been described in detail (56, 83), but briefly a buffer filled fused silica capillary (Polymicro Technologies, Phoenix) with 10 um i.d. and of 65 cm length was placed between two buffer reservoirs. High voltage was applied at the injection end, and the reservoir containing the outlet detection end was held at ground potential. Electrokinetic injection was used for all sample introductions, 5s at 30 kV, sample volume was approximately 600 pL. The easily oxidized analytes were detected

in the amperometric mode with a two-electrode configuration using the optimized end-column detection (84). Carbon-fiber microelectrode was inserted into the end of the electrophoresis capillary and held at 0.8 V versus a sodium-saturated calomel electrode. Reagents: 2-(N-Morpholino) ethanosulfonic acid (MES), serotonin (5-HT), NE, E, DA, L-hydroxylphenylalanine (L-DOPA), VMA, MHPG, HVA and dihydroxyphenylacetic acid (DOPAC) were obtained from Sigma and used in the form received. The electrophoresis buffer was 25 mM MES adjusted to pH 5.65 with NaOH. Calibration standards were prepared as 10 mM stock solution in 0.1 M perchloric acid and diluted to the desired concentration in electrophoresis buffer. Hydrofluoric acid was obtained as a 40% aqueous solution from Aldrich, and used for the etching of the detector end of the capillary.

Catecholamine levels of lymphocytes have been quantified by direct comparison with the standard electropherograms run before and after the patients' sample. The catecholamine content of the lymphocytes is given in fmol/ug protein. Detection limits were determined (for dopamine, norepinephrine 0.13 fmol/ug protein, for epinephrine 0.37 fmol/ug protein, and for DOPAC 0.11 fmol/ug protein) and estimated at twice the peak-to-peak noise level by extrapolation from plots of peak area versus concentration. In between the series of runs, the capillary was flushed with 0.1 M NaOH to refresh the inner capillary surface and to maintain reproducible separation conditions. For more detailed description of the method see Bergquist et al. (55).

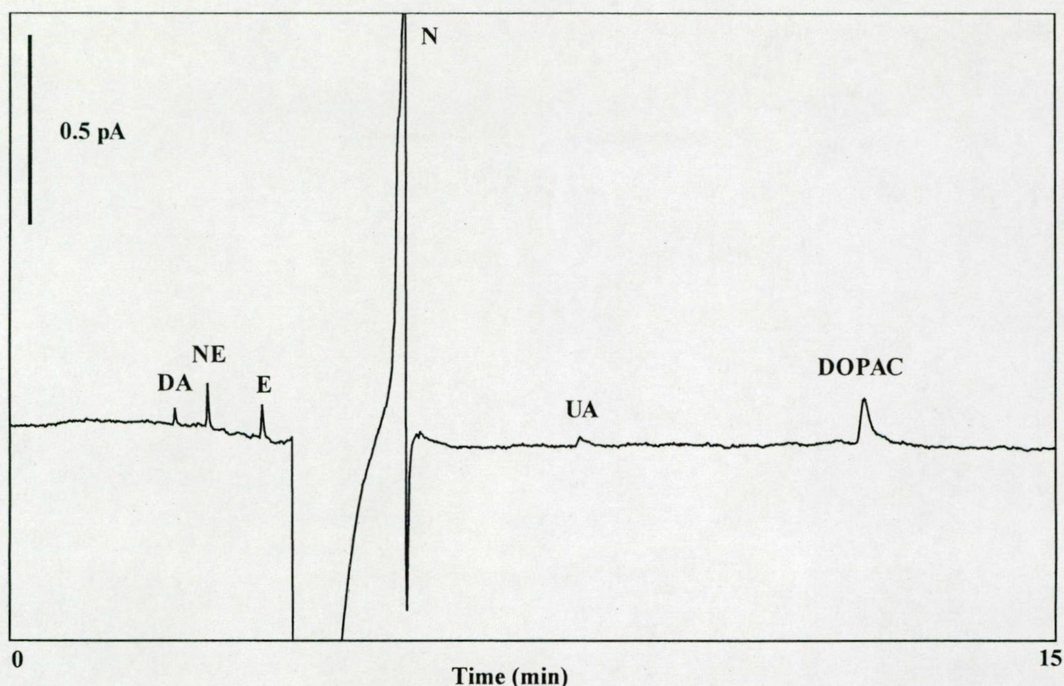

Figure 3. A representative electropherogram of catecholamines extracted from human peripheral blood lymphocytes showing the peaks of dopamine (DA), norepinephrine (NE), epinephrine (E), neutral species (N), uric acid (UA) and dihydroxyphenylacetic acid (DOPAC).

2.3 Statistics

2.3.1. Statistical analysis of the GAMES study (Paper IV)

Since each microsatellite marker was amplified once by PCR and electrophoresed twice, two replicate AIPs were generated from each of the 2 pooled samples. Best fit AIPs were calculated for both the cases and controls, normalized according to the total number of alleles in the respective pools and compared statistically a Chi^2 test. Alleles with a frequency of <5% were considered together. Given that the majority of typed markers are not expected to be associated with MS we were able to use the observed distribution of

Chi^2 results in order to correct the additional sources of variance introduced by pooling. This process was completed using the software package specifically developed for GAMES. The empirical p-values thus calculated enable the typed markers to be ranked according to their evidence for association. Data was analyzed incorporating the adapting factors suggested by Yeo et al. (85). For more details about the statistical analysis see Setakis et al. (86).

2.3.2. Statistical analysis of the intracellular catecholamines (Paper VI)

Kruskall Wallis test (SPSS 7.5 for Windows) was performed for statistical analysis to compare the catecholamine levels in healthy controls and the subgroups of MS patients, followed by Mann Whitney U test for pairwise comparison to see the differences between patients and healthy controls. Furthermore, Kruskal Wallis test was used for the statistical analysis of differences between the healthy controls and the different MS subgroups (regarding EDSS score, medication, and duration).

3. Results

☑ Epidemiological data were evaluated based on the patients data cared at the MS Outpatient, Unit Department of Neurology, University of Szeged, Hungary. We calculated the prevalence, incidence, and the percentage of the different clinical forms in the city of Szeged, and the county Csongrád (Paper I and II). The data are shown in Table 1.

Our data are comparable to the international data, and are a reliable background for planning other studies where MS patients are involved.

Table 1. Comparison of the epidemiological data in the city of Szeged and Csongrád county

Epidemiological data	City of Szeged	Csongrád county
Prevalence	65/100000 (Dec. 31, 1996)	62/100000 (July 1, 1999)
Male: female ratio	1:3	1:2.75
Mean age at onset (all forms)	35 years	29.5 years
Clinical course		
-benign	4%	15%
-RR	80%	54%
-SP	4%	20%
-PP	11%	11%
-RP	1%	NP
EDSS	All forms (N=130)	RR patients (N=82)
EDSS (0)	21%	27%
EDSS (1-4)	57%	EDSS (0-4): 60%
EDSS (4.5-6.5)	16%	33%
EDSS (=7)	2%	NP
EDSS (≥7)	4%	7%
Incidence in Szeged	7/100000 (1996)	
	5/100000 (1997)	
	6/100000 (1998)	

NP- data not published

☑ The CSF sampling was standardized, new laboratory sheet was introduced, the samples were provided with uniform encoded labels, and the CSF bank has an updated register.

☑ In MS patients, the oligoclonal bands, protein content, nephelometry and presence of variant specific antibodies showed a considerable correlation (Table 2). Predominance in the positivity of IgG (6/9=67%) and IgM (4/9=44%) to HHV-6B over IgG (4/9=44%) with no detectable IgM to HHV-6A was found. This raises the possibility that past HHV-

6A infection silent at time of test, and chronic active or primary HHV-6B infection (especially in cases 2, 5, 6, and 7) might contribute to MS. On the contrary, lack of antibodies in CSF to HHV-7 can exclude its role in MS. As seven of nine patients had antibodies to HHV-7 in their sera (data not shown), this also demonstrates integrity of BBB. No antibodies to HHV-6 and HHV-7 were found in the youngest MS patients (1 and 5), which might suggests involvement of another agent(s) in triggering disease. In other neurological disorders, CSF contained no detectable antibodies to HHV-6 or HHV-7. In patient 13, HSV-1 was identified as a causative agent of encephalitis. In the serum and CSF of MS patients we found HHV-6A viruses more often. These data suggest that latent virus replication as environmental factor might be implicated in the induction or pathogenesis of the disease (Paper III).

Table 2. Antibodies to HHV-6 and HHV-7 in CSF of patients with MS and other neurological disorders (ELISA)

No.	Age Years	Sex M/F	Clinical form	Leukoc 10^6/l	Protein g/l	Nephelometry	OCB	HHV-6 IgG	HHV-6 IgM	HHV-6 IgG	HHV-6 IgM	HHV-7 IgG	HHV-7 IgM
										Antibody titres			
1	25	M	RR	1	0.26	-	+	0	0	0	0	0	0
2	29	M	RR	45	0.38	IgM, IgG	+	0	0	800	400	0	0
3	34	M	RR	0	0.22	IgA, IgM, IgG	+	400	0	100	0	0	0
4	53	M	RR	1	0.54	IgG (traces)	+	0	0	100	0	0	0
5	34	F	RR	0	0.26	IgG	+	0	0	200	200	0	0
6	36	F	FA	0	0.41	IgA, IgM	-	200	0	200	100	0	0
7	41	F	RR	5*	0.20	IgG	+	400	0	800	200	0	0
Other neurological disorders													
8	42	M	IDR	0	0.40	IgM	-	0	0	0	0	0	0
9	43	M	CHA	0	0.39	ND	-	0	0	0	0	0	0
10	50	M	CHA	1	0.30	-	-	0	0	0	0	0	0
11	31	F	AHE	100**	0.86	IgA, IgM, IgG (traces)	-	0	0	0	0	0	0
12	25	F	PNP	0	0.17	IgM, IgG	+	0	0	0	0	0	0
13	22	F	PNP	21	0.47	IgG	+	400	0	0	0	0	0

RR = relapsing-remitting; FA = first attack; IDR = intervertebral disc rupture; CHA = chronic headache; AHE = acute HSV-1 encephalitis; PNP = polyneuropathy; ND = not determined; *64 and **104 erythrocytes (10^6/L), 0 = <100.

☑ In an international genome-wide screen for association in MS, the susceptibility to MS in Hungarian patients was studied. In the Hungarian population we have identified 33 potentially associated markers. Six of the markers were from regions previously identified by linkage analyses: D3S3571, D5S2102, D7S2516, D7S630 (87), DXS6807 (88) and DXS1223 (89) and the rest were from novel regions not previously identified (Paper IV, Table 3).

Table 3. Empirical p-values for the top 20 markers.

Locus	Chr	p-value	Mb	*Locuslink	*localisation
D16S3097	16	0,006	78,35		
D7S630[a]	7	0,013	86,98		
D19S921[a]	19	0,015	54,13	VN1R4	19q13.42
DXS6807	X	0,017	3,57		
D6S275	6	0,019	93,03		
D9S1853	9	0,02	30,03		
D2S338	2	0,022	235,92		
D3S1283	3	0,029	28,21	Marfan sy	3p25-p24.2
D14S281	14	0,029	48,9	KIAA1053 protein	
D20S892	20	0,03	6,7		
D18S1127[a]	18	0,032	53,27		
D6S424	6	0,035	95,49		
D3S3571[a]	3	0,036	63,79		
D7S2516	7	0,037	69,75		
D8S1762	8	0,038	101,53	Cohen sy 1	8q22-23
D1S2677	X	0,038	46,1		
D20S891	20	0,04	45,62	PKBP1	20q13.12
D10S1669	10	0,042	43,59		
D5S502	5	0,043	26,12		
D5S2045	5	0,044	169,03		

[a] These markers were included in the 529 considered by Yeo et al.

*data found at the www.nih.gov website for these markers

☑ We reported a case of multiplex MS family with three affected sisters. In this unusual case all the two parents and four grandparents were healthy, without any neurological disorder (Paper V).

☑ Using capillary electrophoresis with electrochemical detection catecholamines were measured in the peripheral blood lymphocytes of RR MS patients. First attack MS patients had higher intracellular epinephrine levels (p=0.028) compared to the controls, while decreased norepinephrine concentration (p=0.027) was detected in the stable RR MS group

compared to first attack MS patients. The increased epinephrine level might be due to the activated immune system. The smaller amount of norepinephrine in the PBL of the stable RR MS group suggest a clinically and immunologically stable phase of the disease (Paper VI). These findings are in line with our other study (Rajda et al. ECTRIMS 2001, abstract).

☑ Finally we reported our experiences of immunomodulant therapy (interferonβ-1b) in a three-year follow-up open labeled study, where the relapse-free proportion was higher than in the previous placebo-controlled studies (Paper VII). This was in line with other published open-labeled studies (Table 4).

Table 4. Differences observed during the 3 years of IFNβ-1b treatment (mean±SD)

	1995	1996	1997	1998	1999
No. of relapses / patient	1.0±0.5	1.6±0.6	0.4±0.5	0.4±0.4	0.2±0.4
Days of hospitalization / patient	14±10	18±11	3.5±6.8	3.1±5.2	0.7±2.1
Need of steroid (g)/patient	4.3±2.8	7.0±2.5	1.7±2.9	1.7±2.4	0.6±1.6

Data given as mean±SEM

4. Discussion

4.1 The role of epidemiological findings

Epidemiological studies are of great help in finding etiological factors in different disorders, such as multiple sclerosis. The diagnostic criteria of multiple sclerosis have recently improved, and the prevalence of this disease can now be determined more precisely. Epidemiologists in many countries worldwide were reanalyzing the prevalence of multiple sclerosis (7-9, 90-99). These studies highlighted both environmental and genetic factors implicated in this disease. Among Japanese patients Devic's neuromyelitis

optica is more common than among the Caucasian patients pointing to genetic predisposition (100), while the seasonally increased MS incidence at Faroe Islands suggest environmental influence in the pathogenesis of the disease (97). Our findings in Paper I and II are in line with the findings of other epidemiological studies at the same latitudes, and made our data comparable to the literature.

4.2. Environmental factors – the role of viruses

Both HHV-6 (101) and HHV-7 (102) induce intrathecal synthesis of IgM and IgG. Until the breakdown of BBB in the later stage of disease, variant specific antibodies primarily in the CSF (101) and secondarily in the serum (32, 103-105) might indicate disease progression. Significant correlation was reported between levels of soluble CD46 (cellular receptor for HHV-6) in the serum and cerebrospinal fluid of multiple sclerosis patients (106). Presence of CSF antibodies in different titers suggests different roles of HHV-6 variants, while the absence of CSF antibodies to HHV-7 seems to exclude its intrathecal contribution to the pathogenesis of MS. In few cases, detection of low level IgG without IgM to HHV-6 indicates past infection without recent expression of viral genes. We found HHV-6A antibodies more often in MS patients than controls suggesting a possible role of these viruses in the initialization or in the pathogenesis of the disease.

4.3. Genetically determined susceptibility

Three independent genome screens typed a set of microsatellite markers covering the genome. Only 43 markers were common to all the three screens (107). In our study, from the 33 associated markers only six were from regions previously identified by linkage analyses: D3S3571, D5S2102, D7S2516, D7S630 (87), DXS6807 (88) and DXS1223 (89) and the rest were from novel regions not previously identified. Large population-based

studies and populations containing the affected genes more often (multiplex families), than the general populations are needed to find the genes of interest in MS. Careful descriptions of familial aggregation and collection of data and DNS samples from these families could be helpful in searching for the cause of genetic susceptibility in MS.

4.4. The role of norepinephrine in the immune responses

Alterations have been described in sympathetic nervous system regulation of immune function, including decreased serum norepinephrine levels in RR MS patients in relapse (108), higher baseline serum levels of dopamine, norepinephrine and epinephrine in RR MS patients (109), elevated norepinephrine levels in the CSF (110), and altered ic. norepinephrine levels (109, 111). The increase in catecholamine levels due to physical stress were similar in MS patients as compared to healthy controls (112). The activation of the sympathetic nervous system seems to be associated with clinical activity in RR MS (112, 113), while the norepinephrine in the plasma revealed to be biochemically stable in healthy volunteers (114). In vivo evidence for an immunosuppressive role of norepinephrine is based on animal and neuropathological studies. Norepinephrine modulates inflammatory gene expression in vitro, inhibiting the expression of genes of NOS2, adhesion molecules, class II MHC, IL-1β, TNF-α (as reviewed by Feinstein, 2002) (115). This evidence provides additional support to the idea that the CNS can modulate inflammatory and immune reactions both centrally and in the periphery. The inhibitory actions of norepinephrine may be mediated primarily within the nucleus affecting the NFκB : IκB system (115). Based on numerous studies, norepinephrine may regulate early immune events as antigen localization, presentation, B cell activation or inhibition of T suppressor cell activation and regulate both Th1 and Th2 cell function (116, 117). Norepinephrine may also suppress normal immune response (51). Elevated levels of

norepinephrine were observed in CSF but not in the blood of MS patients (118). A hypothesis states that there is a deficiency of norepinephrine in the nerve terminals in MS similar to the dopamine deficiency in the Parkinson disease patients. This hypothesis is supported by the fact that near the fourth ventricle lies the locus ceruleus, a norepinephrine mediated part of the brain regarded as a "stress center". Lower levels of norepinephrine in MS could maybe explain the reduced awareness and memory function, difficulties with miction and cerebellar symptoms which are the opposite of the "fight or flight" reactions (119). We observed higher intracellular levels of epinephrine in first-attack MS patients, and the lymphocytes express primarily β-adrenergic receptors. Thus, we can propose the following hypothesis, presented schematically in Figure 5. An increased level of E activates the lymphocytes; they cross the BBB and find their antigens. This process is followed by the production of cytokines, which either result in an inflammatory process or act as the major compartment in the relapse process. A relapse-increased β-receptor density on the lymphocytes has been described, lending support to our hypothesis. It is not clear whether the lymphocytes merely mirror the state of the disease, reflecting the altered hypothalamus-pituitary gland-adrenal medulla (HPA) axis function and drain the catecholamines from the plasma, or are active participants, eliminating the catecholamines by uptake and degradation or releasing them into the MS plaque. The lower level of NE in the peripheral blood lymphocytes of RR MS patients in remission could be due to the β-adrenergic receptor down-regulation after a bout or to the degradation of the catecholamines. Remission may be due to a general down-regulation of the immune response by immunologically nonspecific mechanisms, such as the endogenous secretion of corticosteroids. Later in the disease process, a negative feedback suppresses the production of the catecholamines, resulting in a decreased catecholamine content of the peripheral blood lymphocytes during remission. This may explain why RR MS patients in

remission may have lower levels of catecholamines such as NE and also account for the neuroimmunological entity of the relapse. Relapses can be induced by infection, stress, or an elevated level of E, which activates the lymphocytes, resulting leading in turn to activation of the disease. MS patients have a significantly lower NE content in their peripheral blood lymphocytes than that for healthy individuals, but in the early stage of the disease, and hence in first-attack patients, the E content is higher. With regard to the fact that the lymphocytes in relapse have a higher β-receptor density, new means of early intervention in the pathogenesis of MS at the lymphocyte level may be possible.

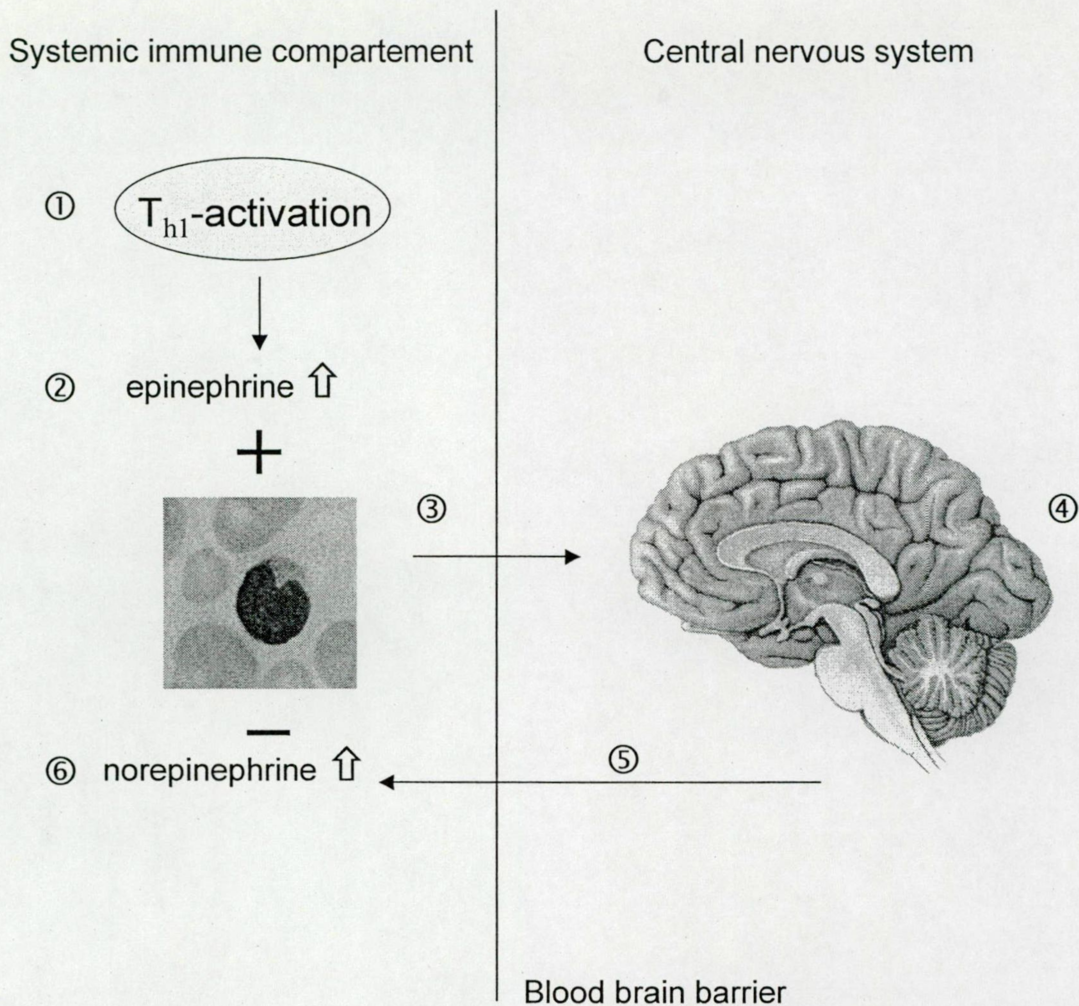

Figure 4. A hypothetical role of the catecholamines in the pathogenesis of MS. ① Th1 T cell activation takes place in the periphery. ② Increased epinephrine levels activating the lymphocytes augment their entrance through the BBB.③ Well inside the CNS, the lymphocytes find their antigens and are further activated. ④ After the activation of the lymphocytes, a feedback process is initiated. ⑤ This feedback loop leads to lymphocyte deactivation and the epinephrine content of the lymphocytes is decreased. This epinephrine decrease is then followed by an increase in norepinephrine, causing a down-regulation of the lymphocytes ⑥, and leading to the steady state of remission.

4.5. IFNβ-1b therapy in RR MS patients

IFNβ-1b (Betaferon ®) is a non-glycosilated recombinant human β-IFN produced in *Escherichia coli* bacteria, administered every other day sc. in 8 MIU dosages. At the double dose given to healthy volunteers, serum concentrations reached a peak of 40 MIU/mL within 1 to 8 hours, with a bioavailability of 51% (120). The serum concentrations were observed to peak 8 to 24 hours after sc. administration of 8 MIU (120 to 475 IU/mL) in patients with MS. In an open-label follow-up study we reported on sustained benefit of the immunomodulant treatment, which was in line with other open-label follow-up studies (121).

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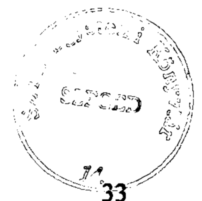

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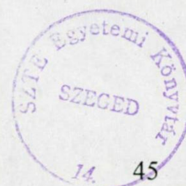

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